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Metabolites of the Wood Rotting Fungus *Fomes roseus*

by

Kanwal Krishen Dhar

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A THESIS

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Date Sep 26, 1985

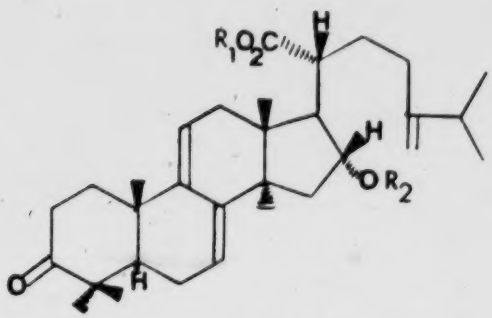
Dedicated to my beloved parents, Mr Sri Kanth Dhar and Mrs Lakshmi Dhar

ABSTRACT

Fomes roseus (Alb. and Schw. ex. Fries) Cooke, is a wood rotting fungus found in the Province of Alberta predominantly on *Picea* (spruce) trees. We have investigated some of the metabolites produced by this fungus, both in nature and in liquid culture. The metabolites isolated and characterized from the malt culture broth are linoleic (41), p-hydroxyphenylacetic (42), and p-hydroxybenzoic (43) acids. These acids have not previously been reported from *Fomes* species. No triterpenoid metabolites were found in the malt culture broth of *Fomes roseus*.

The compounds isolated and identified from the sporocarp include squalene (1), linoleic acid (41), an unidentified triglyceride (44), ergosterol (45), ergosterol endoperoxide (46), polyporenic acid-C (47) and dehydrotumulosic acid (48). The two triterpenoid acids have been derivatized and chemically correlated. They have not previously been reported from any species of *Fomes*. Conflicting and incomplete reports concerning polyporenic acid-C and dehydrotumulosic acid, the result of contamination by the Δ^{8}-congener and other closely related inseparable compounds, required a detailed study of these acids in order to assign the structure with certainty. The oxidation of polyporenic acid-C to the corresponding ketone using tetrakis (pyridine) silver dichromate has been carried out. To our knowledge this is the first report of an oxidation of a secondary alcohol by this reagent and an

improved work up procedure has been developed for this oxidation. The results of antibiotic biassays of various impure metabolites, pure natural products, and their analogues are described.



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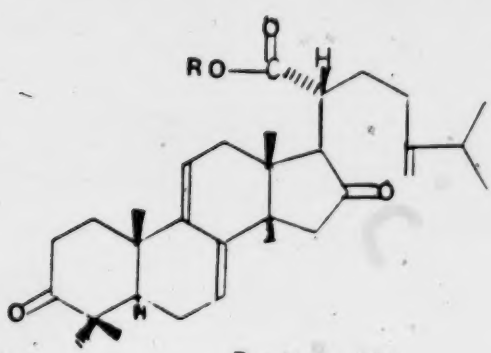
$R_1 = H$	$R_2 = COCH_3$	47b
$R_1 = CH_3$	$R_2 = COCH_3$	47c



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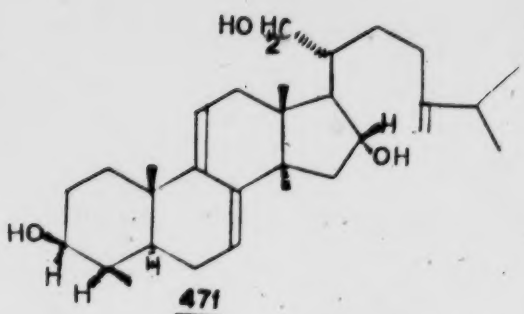

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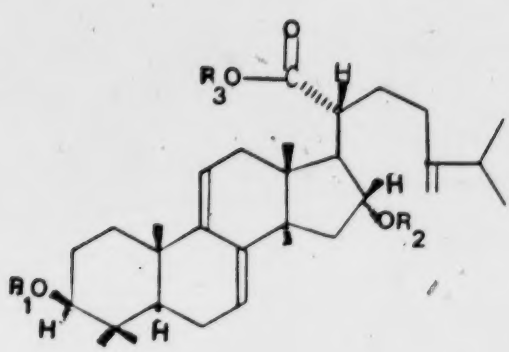

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1. INTRODUCTION

Our forests are an important natural resource. Wood is used as construction material and as a source of paper, chemicals, combustibles and energy. Since decay of wood and control of forest diseases are matters of great economic importance, an understanding of the chemistry of substances produced by wood rot is desirable.

Apart from bacteria, wood rot is brought about by a large number of fungi, which, with only a few exceptions, e.g., *Xylaria polymorpha* from Ascomycetes, belong to the class Basidiomycetes(1). The following families of Basidiomycetes are especially important: Agaricaceae, Hydnaceae, Thelephoraceae and Polyporaceae.

The reported Polyporaceae of North America are comprised of 8 genera, 235 species, and 12 varieties. *Fomes* is the second largest genus of the family. The Polyporaceae also include the genera *Polyporus*, *Lenzites*, *Cyclomyces*, *Daedalea*, *Hexagona*, *Trametes*, and *Favolus* . The most obvious difference between the genus *Fomes* and the largest genus of the family, *Polyporus*, is that the basidiocarp of the latter is annual and that of the former is perennial.

The genus *Fomes* manifests itself in over 47 species and varieties; *F. annosus* , *F. allardii*, *F. applanatus*, *F. fomentarius*, *F. juniperinus*, *F. officinalis*, *F. pini*, *F. robiniae*, *F. senex*, *F. fastuosus*, and *F. roseus* being the more common species. The taxonomic status of *Fomes roseus*

(Alb. and Schw. ex. Fries) Cooke is summarized in Table 1.

Fomes grow in various habitats and this reflects the fact that the genus can adapt to varying environments in nature. The diverse mode of nutrition (parasitic and saprophytic) enables members of the genus to grow on a wide variety of host material.

Fungi which inhabit wood may be broadly divided into a number of groups based upon the nature of their development in and on the wood and upon the type of changes or deterioration for which they are responsible. These groups are listed below:

a) wood destroying fungi, the most economically important group, including the three types of wood rot,

b) wood staining fungi,

c) moulds.

The development of the decay producing fungi depend upon a number of factors:

a) suitable food supply,

b) sufficient moisture,

c) small amounts of air, and

d) favorable temperature.

Deficiency in any one of these requirements will inhibit the growth of the fungus. The fungus secretes enzymes and other organic compounds thereby breaking down the wood cell wall constituents and transforming it into food.

The composition of wood cells consists mainly of cellulose and lignin. The actual effect of the fungal attack

Table 1

Taxonomic Status of *Fomes roseus*

Group	Fungi
Class	Basidiomycete
Subclass	Homobasidiomycetidae
Order	Agaricales
Family	Polyporaceae
Genus	*Fomes*
Species	*roseus*

will vary with the substance which is affected. The cellulose component of wood is light in colour, soft, tough, and has a great affinity for water; while the lignin component of wood is dark in colour, hard, brittle, and has little affinity for water.

Wood rotting fungi play an ecologically important role by causing the decay of wood, thus promoting the cycling of elements otherwise held in an inert and unavailable form. The three type of rot associated with the wood rotting (destroying) fungi are the following:

1) white rot (lignin decomposers of wood)

2) brown rot (cellulose decomposers of wood)

3) hole or hollow rot (lignin and cellulose decomposers of wood).

Brown rot sometimes occurs after white rot in the same tree but white rot seldom follows brown rot. Wood rot may also be classified as root, butt, stump, trunk and top rot to indicate that portion of the tree which is attacked.

Fomes roseus , a type of brown rot most frequently found on the top (branches, stem) of the trees, is commonly called "Brown top rot". *Fomes roseus* grows widely in this province on trees of the genera *Picea*, *Pinus*, *Larix*, *Abies*, *Juniperus*, *Pseudotsuga*, *Tsuga* and is occasionally found on hard woods such as *Betula*, *Populus*, and *Prunus* . However, most of the *Fomes roseus* causing wood rot in Alberta is found on *Picea* (2).

Fomes roseus has been reported to occur in coniferous and

mixed forests of North America(3,4), Mexico(5), Japan(6), Britain(7), Scandinavia(8) and the European U.S.S.R and Caucasia(9).

The fruiting body of this fungus is called a basidiocarp or in general terms a sporocarp. Foresters usually refer to it as a "conk". The basidiocarp is sessile, convex in shape, and ranges from 1.5-10.0 cm in diameter, 0.9-7.0 cm in height and 1.3-3.0 cm in depth. The pileus surface of the sporocarp varies from pale cream to brown to charcoal black. The context is pink or rose in colour. The tubes show varying degrees of stratification and a rose - coloured hue similar to the context. The sporocarp occurs either alone (solitary) or in groups on the substratum. These are woody to corky, and never coriaceous as are the basidiocarps of a similar species, *Fomes cajanderi*. The wide variation in the appearance of the field specimens of *F. roseus* can be attributed to environmentally induced variation in the pattern and intensity of pigment deposition between the hyphae of the basidiocarp causing a bewildering range of colouration and texture.

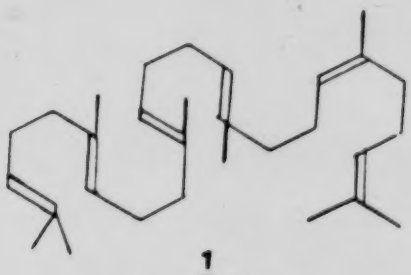
Basidiocarp morphology in nature and in culture is used as a criteria for identification of various genera and species. In cases of ambiguous identification, this method can be supplemented by chemotaxonomical methods. The comparison of thin layer chromatograms of the extracts of various species in resolving taxonomic problems in Basidiomycetes is in vogue (10-15). Currah (2), following a

chemotaxonomical approach, has reported that *F. roseus* and *F. cajanderi* are more closely related to each other than to other species and should be placed in one taxon. His conclusion was based upon ultraviolet spectral data and colour reactions of the fungal extracts. His clarification of the taxonomic status of the two species from a chemosystematic point of view is speculative. More valuable chemical information can be obtained if the organic metabolites of the two species under discussion are investigated and identified. Such an approach to producing taxonomically useful chemical profiles has recently been attempted (16, 17). Our interest in this species arose out of the work of R. Currah of the Devonian Botanic Gardens, who had carried out a preliminary study of the metabolites of *Fomes roseus* (2). Furthermore, early reports (16, 18) of triterpenoids in wood rotting fungi coupled with their structural diversity and biological properties discussed below stimulated us to search for biologically active organic compounds. Such compounds may be important in enhancing our understanding of the chemistry of wood rot. Moreover, the usefulness of these metabolites in the control of forest diseases is a possibility. From the human viewpoint fungal metabolites continue to command interest because of the possibilities of finding new drugs, e.g., cyclosporine. Recently at Michigan State University it was claimed that wood rotting fungi turn toxic dioxins, DDT and PCB's into harmless chemicals (19). A literature review of

chemical compounds isolated from various species of *Fomes* is presented in Table 2.

1.1 Tetracyclic triterpenoids and their biological activity

The triterpenes are part of the family of natural products called "Polyprenoids". They are a group of natural products containing thirty to thirty one carbon atoms in their skeleton. Their structures can be considered as being derived from squalene (1) by cyclization and other



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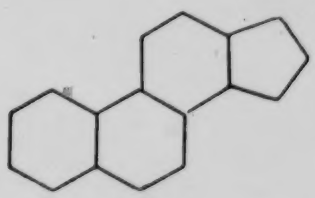

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reactions such as methylation, oxidation, etc. The wide occurrence in nature and the structural diversity of triterpenoids has evoked considerable interest in their biological activity. Recently a number of tetracyclic triterpenes have been isolated and have been shown to possess marked biological effects, e.g., antimicrobial (15), antineoplastic (19), and plant growth inhibition properties (18-23).

Tetracyclic triterpenoids are an important group of compounds and are structurally closely related to the steroids. Most of them contain a perhydrocyclo-

Table 2

Compounds Isolated From Various *Fomes* Species

F. allardii	Cervisterol (25), ergosterol (25), ergosta-5,7,22-trien-3-β-ol (25).
F. juniperinus	Fomecin-A (26) and fomecin-B (26).
F. fomentarius	Fomentariol (27), dehydrofomentariol (28), anhydrofomentariol (29), anhydrodehydrofomentariol (30), ergosta-7,22-dien-3-β-ol (31), 5,6-dihydroergosterol (31).
F. applanatus	(24S)-24 methylcholesta-7, 16-dien-3-β-ol (33), 24 ξ-24 methyl 5-α-cholest-7en-3β-ol (33), ergosta-4,6,8'', 22-tetra-en-3-one (34), and ergosta-7,22-dien-3-one (47).
F. pini	Lanosta-8-en-3β-ol (31), 24-dihydrolanosterol (31), and lanosta-7,9''-dien-3β-ol (31).
F. robiniae	1, 4-dimethoxy-2-nitro-3,5,6 trichlorobenzene (35) and drosophilin-A-methyl ether (35, 36).

F. senex	Pinicolic acid-A (37), senexdiolic acid (37,38), senexanol (34,35), senexdione (37,38), and oxidosenexone (37, 38).
F. officinalis	Dehydroeburicoic acid Ketone (39), 3-Ketedohydrosulphurenic acid (40), eburicodiol (41), eburical (41), officinalic acid (42), polyacetylenes (43), eburicoic acid (39), sulphurenic acid (45), 3β, 6β-dihydroxy-4,4,14α-trimethyl Δ^{8}-5α-pregren-20 one (40), ergosta-4,6,8'⁴, 22-tetraen-3-one (46), eburicol (44), dehydroeburicoic acid (39) and 24-methylene-dihydrolanosterol (44).
F.annosus	(+) - Fomannoxin (48), 6-methyl dehydro-α-lapachone (49), fomajorin-s (50), fomajorin-D (50), 7α, 8β-11-trihydroxydrimane (50,51), ergosta-4,7,22-trien-3-one (31), 5-formyl-3 hydroxy-2 isopropenyl-2,3-dihydro benzofuran (50),

	5-formyl-2-1-(1-hydroxy-1-methyl ethyl)-2,3-dihydrobenzofuran (50), 5-formyl-2-(1, 2 dihydroxy- 1 methyl ethyl)- 2, 3-dihydrobenzofuran (50), 5-formyl-2-(1,2 dihydroxy - 1 methyl ethyl)-2, 3-dihydrobenzofuran (50), hexatriyne (52), ergosterol (31), ergosta-5,7,22-trien-3β-ol (31), ergosta-7,22-dien-3β-ol (31) and 5,6-dihydroergosterol (31).
F.fastuosus	Ergosterol (32), 2,3,5,6-tetrachloro-1,4-dimethoxy benzene (32), and lanosta-8,25-dien-3β-ol (32).

pentanophenanthrene skeleton (2), analogous to that of the steroids but with varying substitution patterns. The main types of tetracyclic triterpenoids are listed in Table 3.

The onoceranes (9) are a group of tetracylic triterpenoids which are grouped separately. Although the onocerins (10) and (11) may appear to be more closely related to pentacylic triterpenes, they are nevertheless tetracyclic. Onocerins have been isolated by extraction from such plants as *Ononis spinosa* and *Lycopodium clavatum* (18).

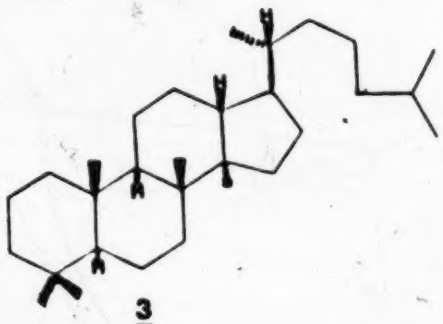
The structural similarity between the tetracyclic triterpenes and steroids together with their wide distribution has prompted the development of a consistent system of nomenclature. Definitive rules for steroid nomenclature have been described (53). The steroid α, β rule is followed in tetracyclic triterpenoids *i.e.*, the substitutents *cis* to the C-19 methyl group are said to be β; substituents *trans* to that methyl group are α. The numbering pattern is presented for the lanostane skeleton (6).

Lanosterol (12) is an emulsifying agent (18) and is widely used as an ointment base in pharmaceutical preparations. Ourisson *et al.* (54) reported that the lanosterol-derived triterpenoids pachymic acid (13), tumulosic acid (14), and their 7,9'' dehydro derivatives obtained from the Chinese drug *Poria cocos* (Polyporaceae) possess cytotoxic activity *in vitro* on cultured hepatoma cells. This cytotoxic activity is also observed with inotodiol (15) and 3β,16α-dihydroxylanost-8-ene (16).

Table 3

Type of tetracyclic triterpenoids

Skeleton	Compound number
Dammarane	3
Tirucallane	4
Euphane	5
Lanostane	6
Cucurbitane	7
Fusidane (Protostane)	8
Onocerane	9



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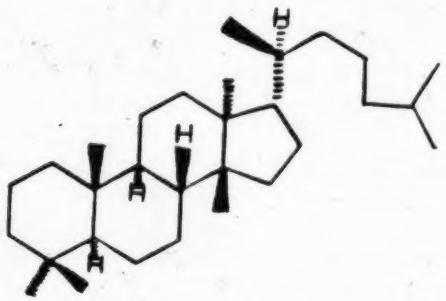

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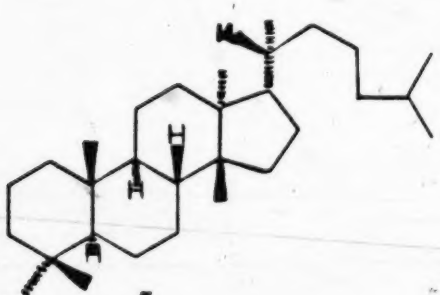

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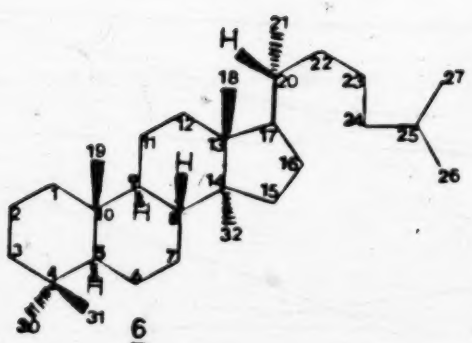

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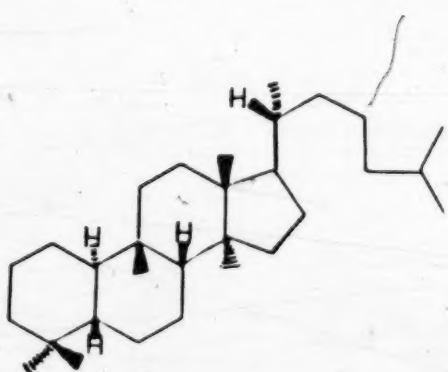

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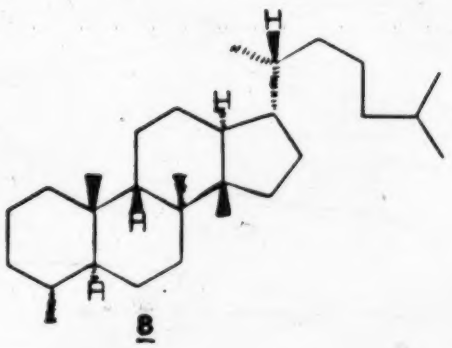

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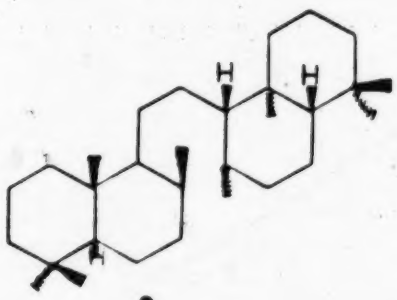

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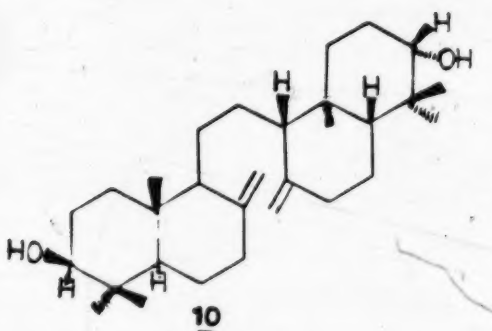

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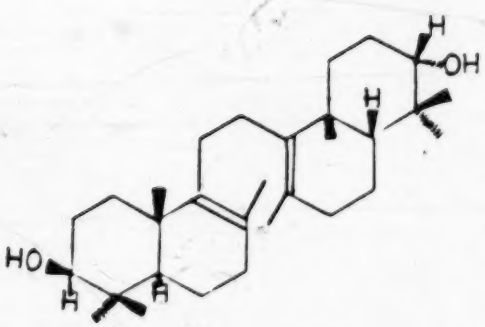

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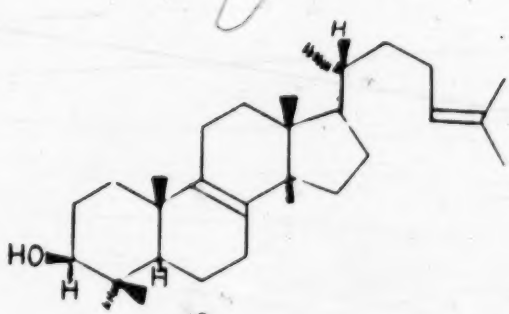
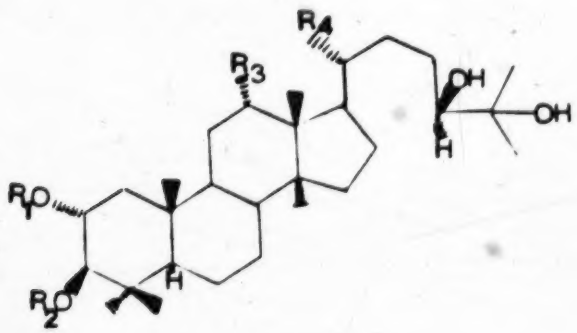


12

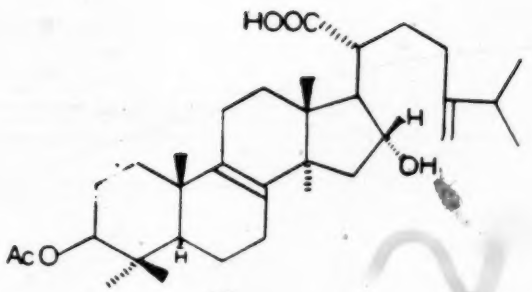
To our knowledge no cytotoxic activity has been reported for lanosta-7,9''diene 3β-ol (dihydroagnosterol, 17). However, the 3-keto- and the 25- or 26-hydroxy derivatives of lanosterol are not cytotoxic. Ikeda *et al.*(55-57) have reported various biologically active lanostanes of *Neamatoloma fasiculare*, for example, fasiculols A-F (18-23).



R_1	R_2	R_3	R_4	
H	H	H	CH_3	(18)
H	H	OH	CH_3	(19)
H	H	OH	CH_2OH	(20)
X	H	OH	CH_3	(21)
H	X	OH	CH_2OH	(22)
X	H	OH	CH_2OH	(23)

Where, X = $CH_3OCOCH_2NHCOCH_2C(OH)(CH_3)CH_2CO$-
(Depsipeptide moiety)

Compounds 18-20 possess the uncommon α-glycol groups at C_{2-3}, C_{24-25}, while compounds 21-23 are triterpene esters possessing the novel depsipeptide moiety and rare 12-α-OH. All fasiculols (19-23) with or without the depsipeptide moiety exhibit approximately the same plant growth inhibitory activity against Chinese cabbage seedlings. The activity of compound 18 was one-fourth that of the other



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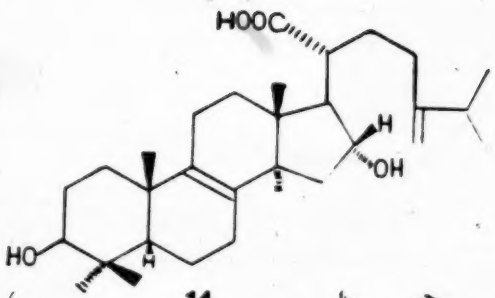

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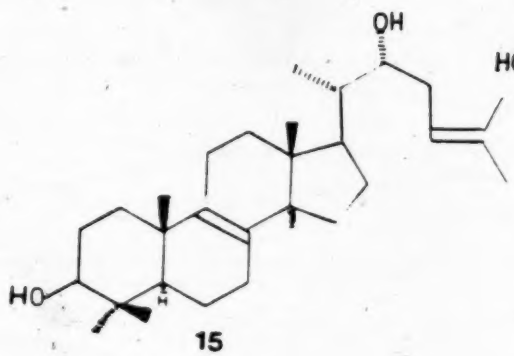

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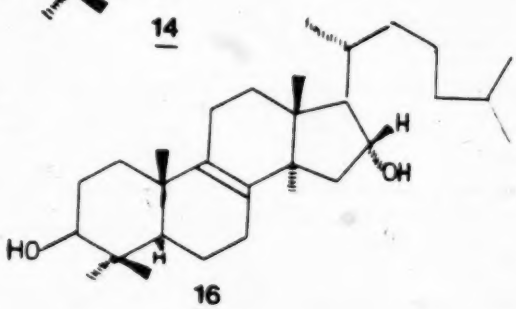

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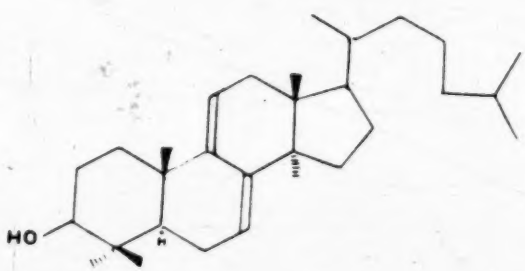

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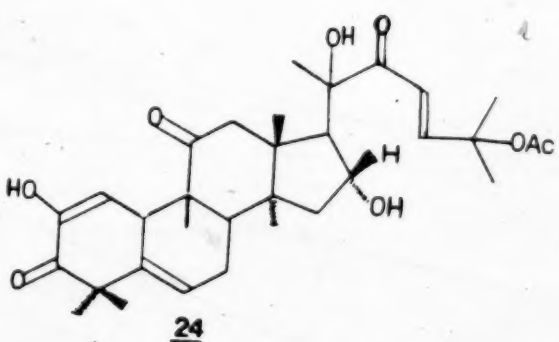

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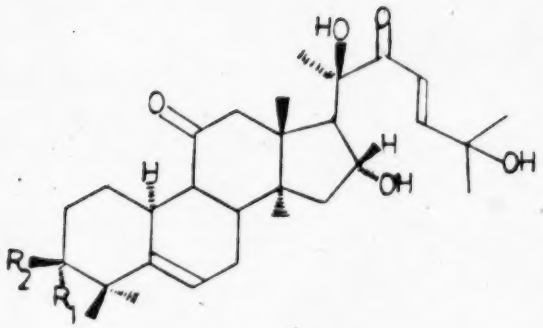
fasiculols (19-23). This suggests that the C-12 hydroxyl group is important for biological activity and that the C-21 hydroxyl group and depsipeptide moiety make little contribution to growth inhibitory activity. The antimicrobial activity of the fasiculols (18-23) have also been reported. To be active against *Staphylococcus aureus* and *Klebsiella pneumoniae* the fasiculols require the presence of the depsipeptide moiety since the fasiculols (18-20) themselves are inactive.

The cucurbitacins present a more varied biological activity. These compounds are acrid, bitter substances present in many medicinal plants, which find use in various traditional Pharmacopoeiae as vermifuges, narcotics, purgatives and emetics, for the treatment of dropsy, dysmenorrhea, malaria and to cure necrotic wounds (18). The cucurbitanes usually occur in nature as glycosides.

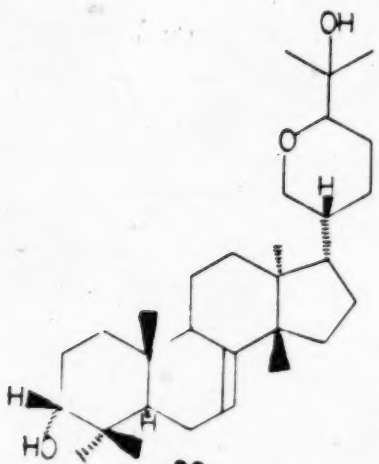
Cucurbitacin-E (24) shows antitumour properties (18). Kupchan *et al.*(59) isolated two new cytotoxic cucurbitacins: isocucurbitacin-D (25) and 3-epi-isocucurbitacin-D (25a), from the dried plant and ground leaves of *Phormiun tenax*. Compounds 25 and 25a showed tumour inhibitory properties against KB cell cultures. Moreover, the latter compound 25a was active against P-388 lymphocytic leukemia in mice. The ethanol extract of dried leaves showed cytotoxicity against cells derived from human carcinoma of the nasopharynx.

Tessier *et al.* (60) studied the toxic African plants of the family Euphorbiaceae and reported that the isolated



R_1=OH, R_2=H **25**

R_2=OH, R_1=H **25a**



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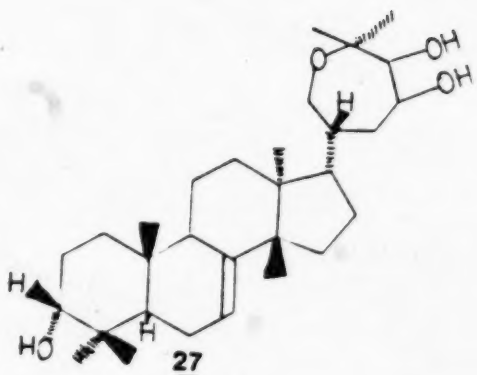

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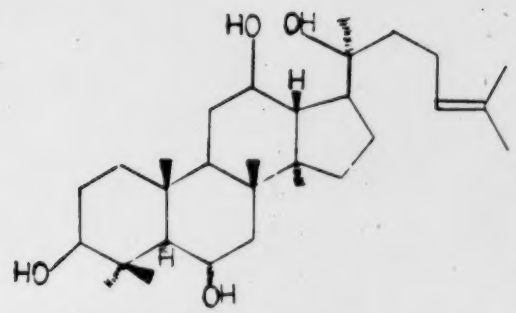
cucurbitacins killed HeLa cell culture within 3 days and inhibited mitosis in *Allium sativum* meristems. Jolad *et al.* (61) observed that the crude chloroform soluble fraction of the ethanol extract of the leaves of *Bursera klugii* (Burseraceae) showed antineoplastic activity against two test systems, the P-388 lymphocytic leukemia (3PS) and human epidermoid carcinoma of the nasopharynx (9KB).
The activity of the extract was shown to be caused by sapelin-A (26) and sapelin-B (27).

Anisimov *et al.* (62) have studied the structure-activity relationship of 35 dammarane-type triterpenoids. Various dammarane triterpenoids, panaxoside-A (28), panaxadiol-3-yl-β-gluco-pyranoside (29) and dihydropanoxoside-A (30), when tested for early embryogenesis of the seaurchin, caused anomalies in the embryo development: notably arrest of egg division and blastomerelysis. In addition compounds 28-30 displayed cytostatic activity.

Physiologically active substances are found not only among tetracyclic triterpenes themselves, but also among certain of their derivatives. Ourisson *et al.* (54) reported that a mixture of Δ^{8} and $\Delta^{7,9(11)}$ (60:40) lanostane compounds, viz., 31 + 31a, 32 + 32a, 33 + 33a, and 34 + 34a showed notable cytoxicity *in vitro* on cultured hepatoma cells.

Fusidic acid (35) is an important antibiotic which is particularly useful for the treatment of staphylococcal



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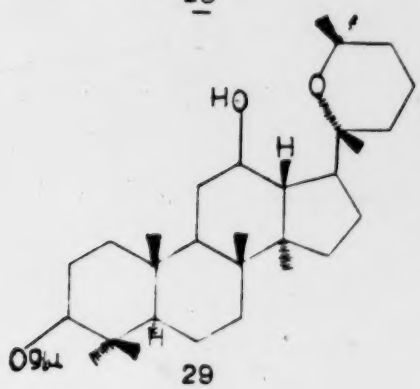

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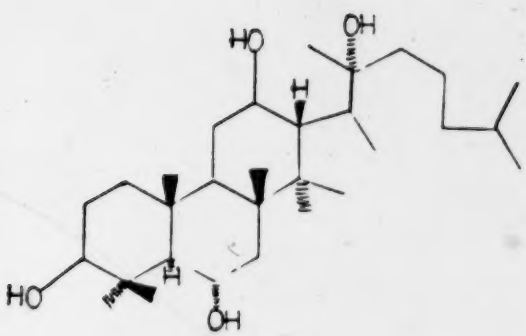

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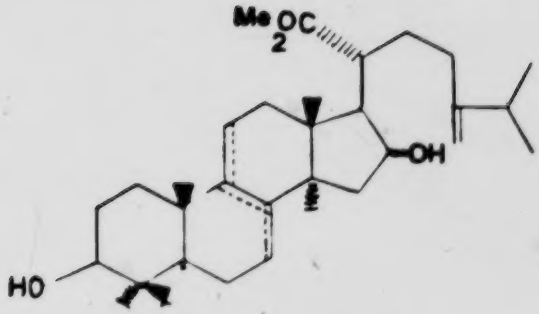
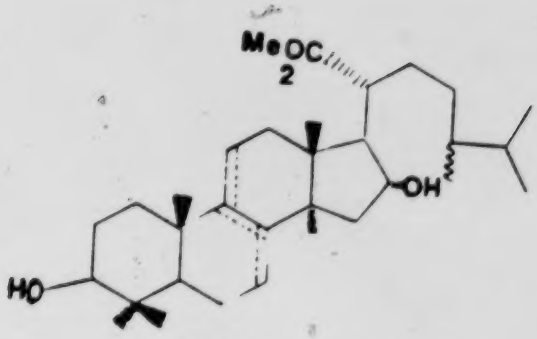
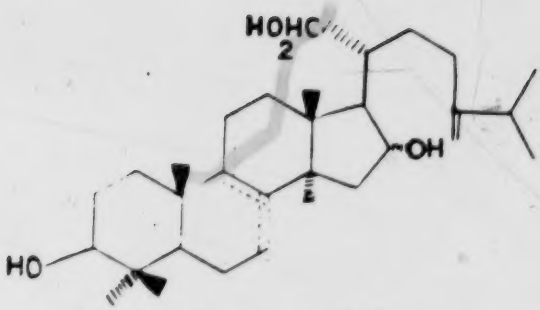


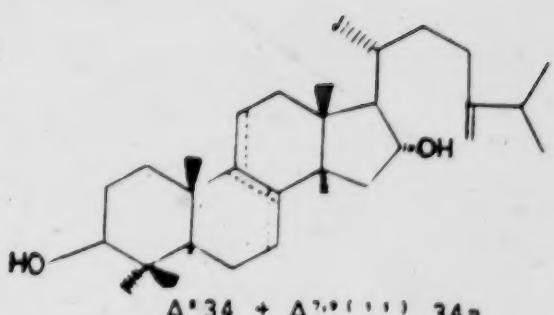
Δ^{9} 31 + $\Delta^{7,9(11)}$ 31a



Δ^{9} 32 + $\Delta^{7,9(11)}$ 32a

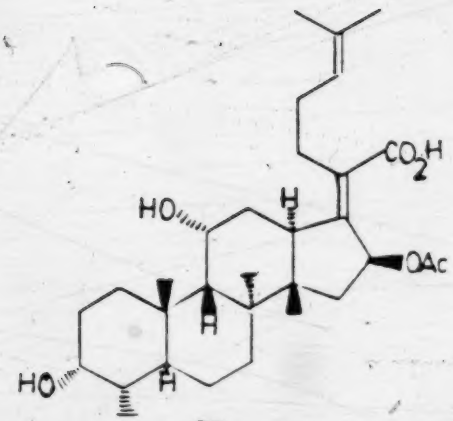


Δ^{9} 33 + $\Delta^{7,9(11)}$ 33a



Δ^{9} 34 + $\Delta^{7,9(11)}$ 34a

infections (63, 64). It is produced by the fungus *Fusidium coccineum* and possesses the unusual *trans, syn, trans* stereochemistry of rings A, B and C. This results in ring B adopting a boat conformation. Fusidic acid blocks active cation transport across the cell membrane (63), a function of $Na^{+}K^{+}$ adenosine triphosphatase. Fucidin, the registered trade mark for the crystalline sodium salt of fusidic acid, is an antibiotic highly active against staphylococcal infections and which does not produce significant toxic effects. Fucidin in combination with other antibiotics have shown synergism and antagonism against various strains of *S. aureus*. Both synergy and antagonism have been shown *in vitro* with the penicillins, and synergy has been shown with tetracycline, lincomycin, clindamycin and other antibiotics. Plasmid mediated and chromosomal mutation resistances have been reported with fusidic acid (65-68). Fusidic acid is active *in vitro* against many gram positive bacteria, *Nocardia, Mycobacterium, Neisseria* species and some anaerobic organisms (68,69). Fusidic acid and its 24, 25-dihydro derivative inhibit polypeptide chain elongation by binding to ribosomes (63). The steroids of the fusidane family resemble bile salts. Encouraged by the biological potential of fusidic acid (35), workers searched for other fusidane triterpenes. New biologically active members of the fusidane class, compounds 35(a-d), 36, 36a, 37, have been isolated (64, 69, 70). W. V. Daehne *et al.* (69) have studied the structure-activity relationships in fusidic acid-type



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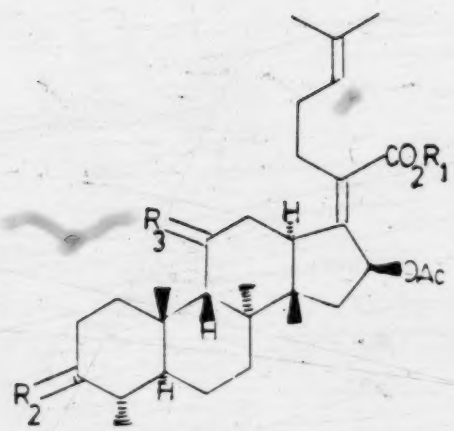
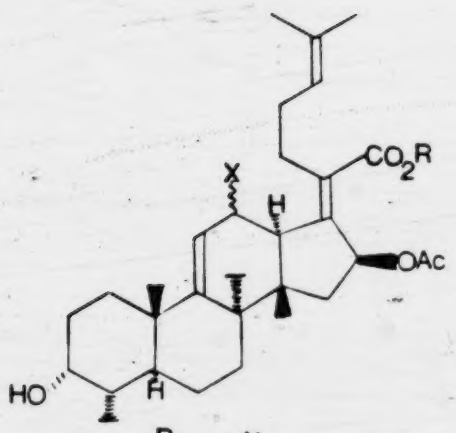
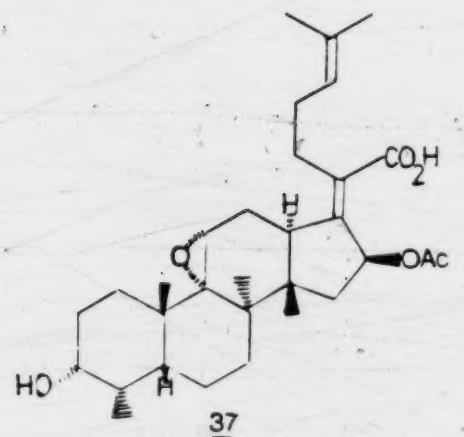


R_1	R_2	R_3	
H	=O	α-OH, β-H	35a
H	α-OH, β-H	=O	35b
H	α-H, β-OH	α-OH, β-H	35c
H	α-OH, β-H	α-H, β-OH	35d



R	X	
H	H	36
H	OH	36a



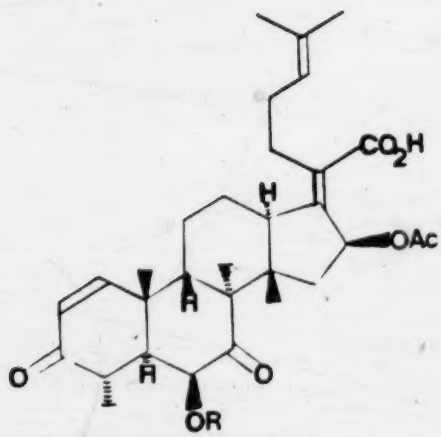
37

antibiotics.

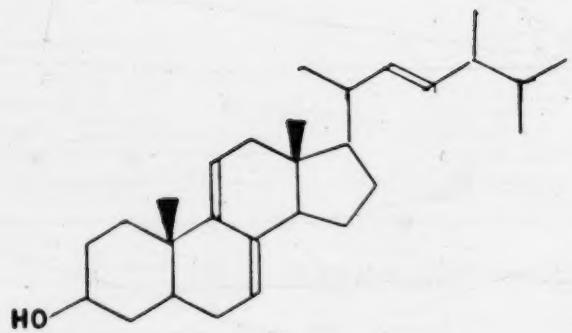
Cephalosporin P_1 (38), helvolic acid (39) and helvolinic acid (39a) have similar antibacterial spectra (64, 65). Fusidic acid (35) inhibits protein synthesis, cephalosporin P_1 (38) does not incorporate into proteins.

Kaise *et al.* (71) have isolated viridominic acids-A, B and C, compounds 40, 40a and 40b, respectively, from a *Cladosporium* species. These metabolites resemble cephalosporin P_1 (38) in that they also possess the interesting biological property of inducing chlorosis in higher plants.

This thesis describes work which has led to the isolation and structure determination of various metabolites isolated from the culture broth and the fruiting body of *Fomes roseus*. As well, the biological activities of various extracts, pure components and their analogues are described.



R= Ac, 39
H, 39a



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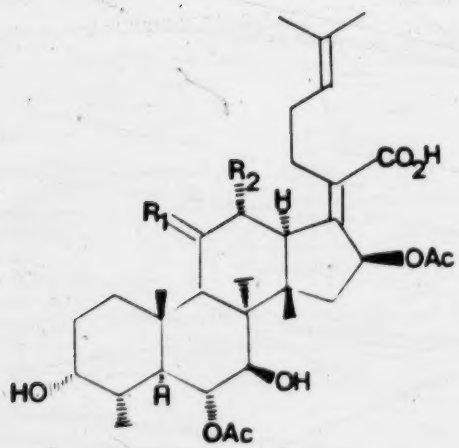
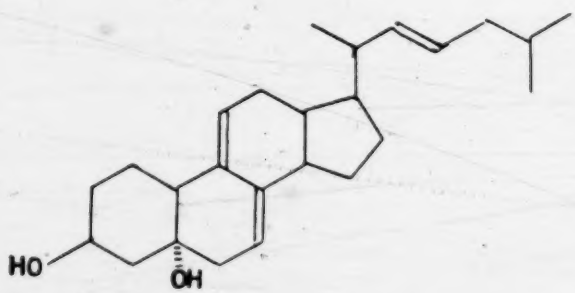


R_1	R_2	
α-H, β-H	H	38
O	H	40
O	OH	40a
α-H, β-OH	H	40b



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2. RESULTS AND DISCUSSION

2.1 Malt culture broth

Fomes roseus (strain B-58, UAMH 4787) was obtained from the Department of Botany, The University of Alberta and identified in the laboratory of Prof. L. L. Kennedy. *F. roseus* was cultured following aseptic techniques in liquid nutrient media to produce large quantities of crude extracts. The medium for the growth of this fungus was selected empirically for a suitable carbon source, a nitrogen source and inorganic ions. Growth factors, such as growth temperature, light/dark conditions and static (still)/shake (fermentor) conditions were selected arbitrarily. Longer incubation is needed as most of the wood rots have a slow growth.

Shukla (72) studied the development of *Fomes igniarus var populinus* in culture and reported that this fungus grows well on malt extract at 27°C for 30 days. This is in agreement with our observation of the growth of *F. roseus* .

The growth of *F. roseus* was carried out in either liquid V-8 medium or malt extract medium in Fernbach flasks for 4 weeks and 7 weeks. Of the two common culture techniques, still and shake, still culture conditions were preferred over the use of fermentor because the still conditions more closely mimic some of the natural conditions of growth for *F. roseus* .

The fungus was harvested by separating the mycelium from the broth. The mycelium was air dried and extracted with ethyl acetate in a Soxhlet extractor. The ethyl acetate was removed *in vacuo* to give the mycelial extracts (170 - 810 mg/L). The broth was concentrated *in vacuo* (5L/9L to 500 mL) and then continuously extracted with ethyl acetate. The ethyl acetate was concentrated to give 0.32 - 1.58 g/L of crude metabolites. The yields of mycelial and broth extracts obtained under various growth conditions are summarized in Table 4. It is clear from Table 4 that the fungus produces larger quantities of metabolites when grown on malt extract than when grown on V-8 extract.

The malt extracts of *F. roseus* were darker in colour and had a different odor than the V-8 extracts of *F. roseus*. Due to the differences in the yield of metabolites, (Table 4), together with the biological activity of the various extracts the malt broth extract was selected for separation studies.

2.1.1 Isolation and identification of metabolites

Flash chromatography of ethyl acetate extracts of 4 weeks old malt broth extract (0.64 g) of *F. roseus* over silica gel gave 14 fractions each of which was a complex mixture when examined by thin layer chromatography (TLC) over silica gel. One of the least polar pinkish-coloured fractions (15 mg) showed a less complex TLC and was selected for attempted purification. The fraction was subjected to

Table 4

Growth Studies on *F. roseus*

Growth medium	Quantity of the medium (liters)	Time of growth (weeks)	Yield (grams)	
			Broth metabolites	Mycelium metabolites
V-8 extract	5	4	0.39	0.49
Malt extract	5	4	0.64	0.38
Malt extract	9	7	1.75	0.90

centrifugal chromatography (CC) over silica gel and eluted with various combinations of Skellysolve B and diethyl ether (3:1, 2:1, 1:1, 100 mL each). A number of fractions (1 mL each) were collected and one of these fractions gave component **A** (1.5 mg) as a yellow oil.

The high resolution electron impact mass spectrum (HREIMS) of component **A** shows a molecular ion at m/z 280 corresponding to the molecular formula $C_{18}H_{32}O_2$[1] and the presence of three unsaturation equivalents.

The Fourier transform infrared spectrum (FTIR) of component **A** shows a broad hydroxyl absorption band (3600-2400 cm^{-1}) and carbonyl (1710 cm^{-1}) absorption characteristic of a carboxylic acid. Weak absorption at 1610 cm^{-1} suggests the presence of olefinic functionality in the molecule.

The proton nuclear magnetic resonance spectrum (^1H NMR) of component **A** displays 4 olefinic protons at δ5.37 as a complex multiplet, four high field methylenes [(δ2.80, 2H, m), (δ2.36, 2H, t, J=6 Hz), (δ 2.04, 4H, m), (δ 1.37, 14H)], and a methyl group (δ 0.94, 3H, vt). This data suggests that component **A** is the common dienoic acid linoleic acid (41). The presence of an acid group at the terminal position of the chain in compound 41 can be demonstrated by the loss of a $C_2H_4O_2$ unit (60 amu) from the molecule giving rise to fragment $C_{16}H_{28}$ in the mass spectrum. This unit and the resulting fragment arise by cleavage β to the carbonyl group

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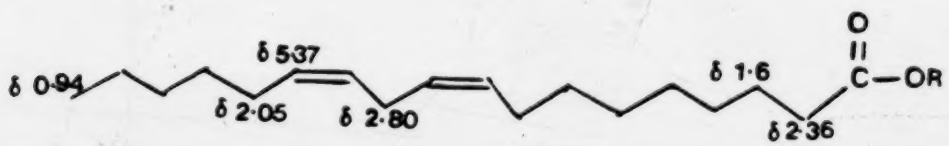
[1]All molecular ions were determined by high resolution electron impact mass spectra.

through a McLafferty rearrangement. Most naturally occurring fatty acids have an even number of carbon atoms and compound (41) is no exception. A comparison of component 41 with an authentic sample of linoleic acid confirmed that they were identical. Treatment of compound (41) with ethereal diazomethane gave methyl linoleate (41a). The 'H NMR signal assignment for linoleic acid (41) is shown on the diagram below.



R = H , 41

R = CH_3, 41a

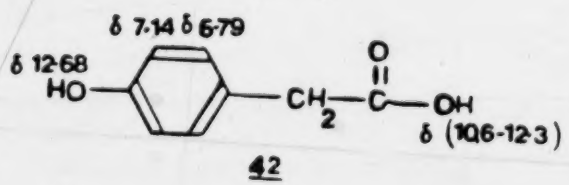
The extract of 7 week old malt broth was taken up in water and extracted with Skellysolve B to remove fatty acids and triglycerides (800 mg). The residual extract was further partitioned with diethyl ether, the diethyl ether extract was concentrated *in vacuo* and then triturated with chloroform. Most of the brownish metabolites dissolved leaving a light brown insoluble residue (50 mg) which was subjected to flash chromatography using polarity gradient elution with various combinations of benzene: diethyl ether and diethyl ether: methanol. The less polar fraction (Rf 0.31, benzene: diethyl ether, 1:1) from the column gave 6 mg of colorless crystalline component B, while one of the less

complex polar fractions contained 10 mg of impure component C. Component C was purified further by flash chromatography.

The HREIMS of component B shows a parent ion at m/z 152 corresponding to the molecular formula $C_8H_8O_3$, and the presence of five unsaturation equivalents. The loss of COOH from the parent ion to give a fragment ion C_7H_7O in the mass spectrum suggests the presence of an acid functionality in the molecule. The HREIMS also shows peaks at m/z 79, 77 which are characteristic of a phenyl ring.

The FTIR of component B shows a broad band 3540-2400 cm^{-1} and carbonyl absorption at 1707 cm^{-1} characteristic of a carboxylic acid functionality. A phenyl ring is further indicated by bands at 1598 and 1518 cm^{-1}.

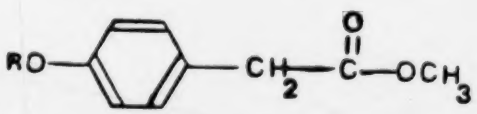
The ^1H NMR shows a low field methylene signal at δ 3.53(s,2H), doublets at δ 6.79 (J=8 Hz,2H) and δ 7.14 (J=8 Hz,2H) indicative of *para* substituted aromatic moiety. Two D_2O exchangeable protons at δ 10.6-12.3 and δ 12.68 were also observed. The spectral data suggests that component B is p-hydroxyphenylacetic acid (42) and that assignment was confirmed by comparison of component B with an authentic sample of 42. The ^1H NMR signal assignment for p-hydroxyphenylacetic acid (42) is shown on the structure below.



42

The mass spectral fragmentation pattern of compound 42 is described in scheme A.

Compound 42 forms a methyl ester, 42a, upon treatment with ethereal diazomethane. Treatment of compound 42a with acetic anhydride and pyridine gave methyl O-acetylphenyl acetate (42b).

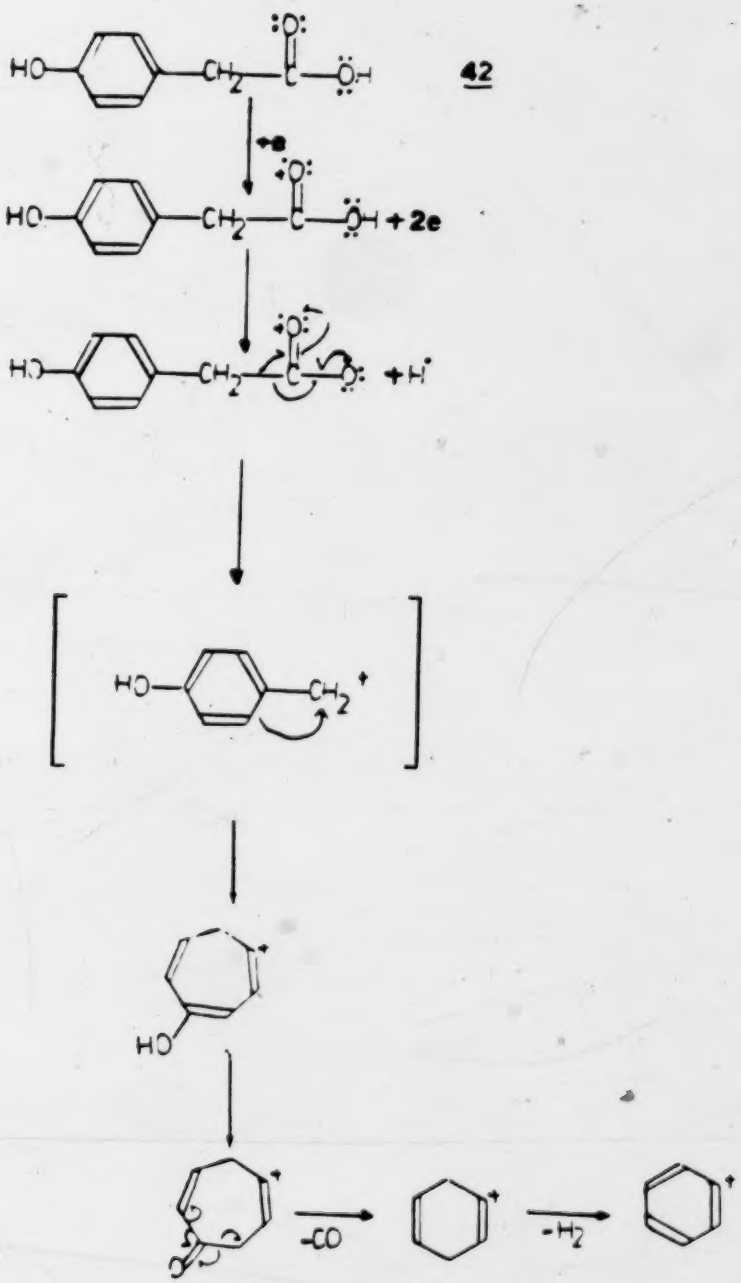


R=H 42a
R=Ac 42b

p-Hydroxyphenylacetic acid (42) has been isolated by Crowden *et al.* (73) from the culture broth of *Polyporus tumulosus*. Compound 42 is used in the fluorometric determination of oxidative enzymes (74) and used recently as the peroxidase substrate in the enzyme immunoassay (EIA) of insulin in serum (75). This is the first reported isolation of 42 from a *Fomes* species.

Further purification of component C gave a white crystalline compound (mp 208-211°C) which is soluble in benzene, diethyl ether, acetone, and methanol.

The HREIMS of component C shows a molecular ion at m/z 138 corresponding to the molecular formula $C_7H_8O_3$, and the presence of five unsaturation equivalents. The loss of a hydroxyl group from the parent ion with the consequent formation of an aromatic acylium ion, $C_7H_5O_2$, is the

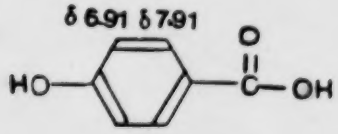


Scheme A : The mass spectral fragmentation pattern of p-hydroxyphenylacetic acid (42)

dominant fragmentation in its mass spectrum.

The FTIR of component C shows a broad hydroxyl absorption band (3600-2000, peak at 3388 cm^{-1}) and a carbonyl absorption at 1674 cm^{-1} characteristic of a carboxylic acid functionality. Further support for an aromatic ring is given by absorption at 1585 cm^{-1}.

The ^1H NMR of component C shows two doublets, one at δ 6.91 (2H, J=8 Hz) and another at δ 7.91 (2H, J=8 Hz) indicating an A_2X_2 spin system. These data suggest that component C is p-hydroxybenzoic acid (43) and this identification was confirmed by comparison with an authentic sample. The ^1H NMR signal assignment for p -hydroxybenzoic acid (43) is shown on the structure below.



43

p-Hydroxybenzoic acid (43) has been isolated by Crowden *et al*. (73) from the culture broth of *Polyporus tumulosus* but has not been reported from any species of *Fomes* . Compound 43 is present in the hydrolysates of certain leaves and has been shown to be an acylating group attached to the constituents of cell walls (76). Monocotyledons have been reported to contain either compound 43 or its glycoside (77, 78). Hillis reported the presence of this acid in lignified

tissues or those containing many dead cells (79).

The components isolated from cultures of *F. roseus* (malt broth extract) are summarized in Table 5.

2.2 Sporocarp

2.2.1 Approaches to the isolation of metabolites from the sporocarp of *F. roseus*

The basidiocarps of *F. roseus* were collected from fallen spruce in August 1982 and August 1983 in Drayton Valley. The basidiocarps were identified by Dr. R. S. Currah, Devonian Botanic Garden, The University of Alberta. The fungus was air dried and wood shavings adhering to the basidiocarps were removed to prevent contamination. The fungus was then subjected to Soxhlet extraction with ethyl acetate. Various attempts were undertaken to simplify and separate the extracts from the sporocarp of *F. roseus*. Initially chromatographic separation of the ethyl acetate extract of the fungus over Sephadex LH-20, followed by slow column chromatography over alumina (Skellysolve B: ethyl acetate) was attempted. The separation proved to be very laborious and inefficient. No purification was possible. The second attempt to separate the fungal extract involved direct slow column chromatography over silica gel using gradient elution with various solvent systems, e.g., Skellysolve B: benzene and benzene: ethyl acetate. This purification attempt also proved futile since the fractions

Table 5

Components isolated from cultures of *F. roseus* (Malt broth extract)

Component Isolated	Time of growth (weeks)	Chromatographic Separation technique
Component A	4	Flash and Centifugal(PTLC)
Component B	7	Solvent partitioning and flash
Component C	7	Solvent partitioning and flash

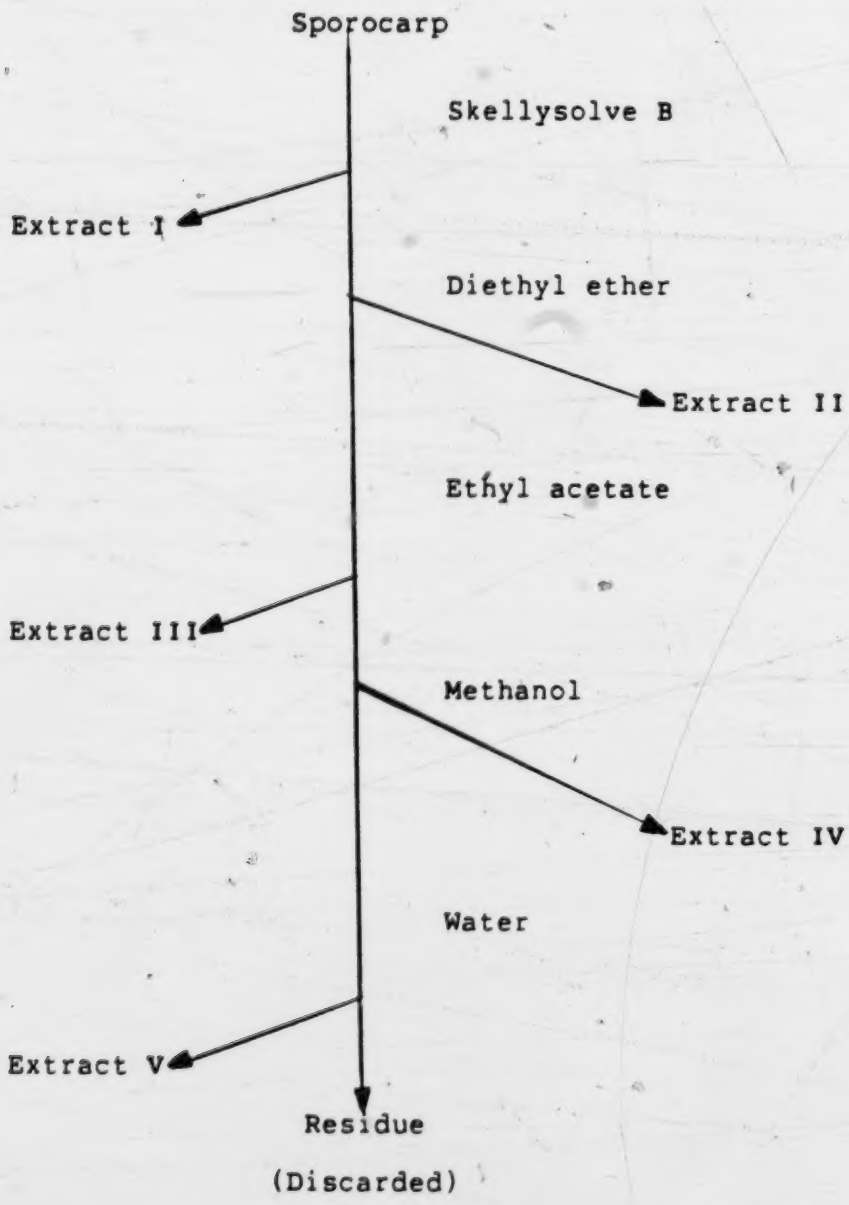
were shown to be complex mixtures by TLC. Because of the complexity of the extracts, preliminary separation by solvent partitioning was attempted. The powdered fungus was extracted successively with solvents in order of polarity to give 5 extracts, viz., Skellysolve B (I), diethyl ether (II), ethyl acetate (III), methanol (IV), and water (V) as shown in Scheme B. Both Soxhlet extraction and solvent extraction using a mechanical stirrer were carried out. These methods yielded comparable quantities of metabolites (Table 6), however, the Soxhlet extraction afforded extracts which were darker in color. The residue remaining after extraction with water was spongy, material which was insoluble in most organic solvents and was discarded. Since the TLC profiles of extracts I-V from Soxhlet extraction and of extracts I-V from solvent extraction by mechanical stirring were identical, the various extracts were combined for further separation studies. Chromatographic separation of the metabolites present in each of the extracts I-V did not appear promising at this point since many compounds (up to 40) were shown to be present when the extract was examined by TLC. Thus further fractionation of extracts I and II by separation into acidic, basic, and neutral fractions was undertaken (Scheme C). Table 6 depicts the quantities of extracts of the sporocarp of *F. roseus* while schemes B and C show the extraction processes employed.

Table 6

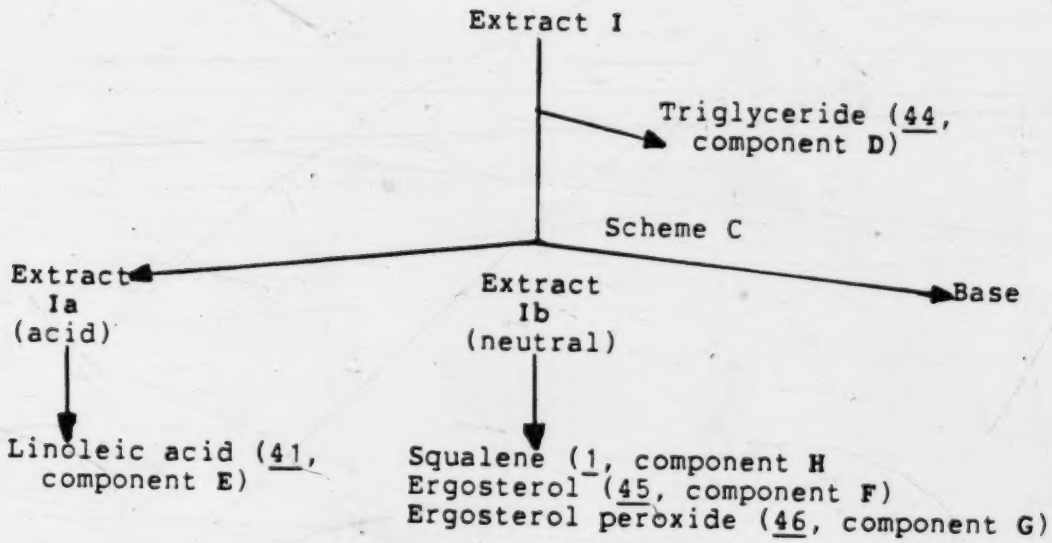
Extracts of crude metabolites from the sporocarp of *F. roseus*

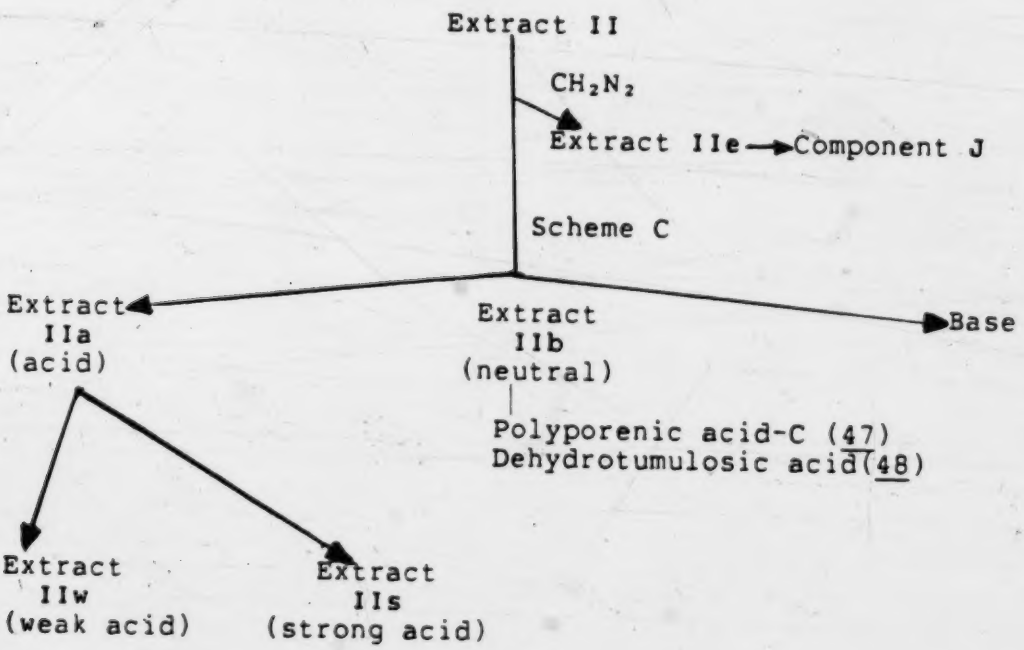
Extracts	Colour	Quantity obtained (g)			Composition of total extracts (g)		
		Soxhlet extraction (from 75g) (S)	Mechanical Stirring (from 75g) (MS)	Total (from 150g) (S+MS)	Strong Acids	Weak Acids	Neutrals
Skellysolve B I	Yellow	4.1	4.2	8.3	4.5	-	3.8
Diethyl ether II	White to yellow	34.0	33.4	67.4	20.1	36.4	10.8
Ethyl acetate III	Brown	2.0	2.3	4.3			
Methanol IV	Dark brown	3.4	3.2	6.6			
Water V	Dark brown	2.5	1.5	4.0			

Overall efficiency of extraction = 60.4%

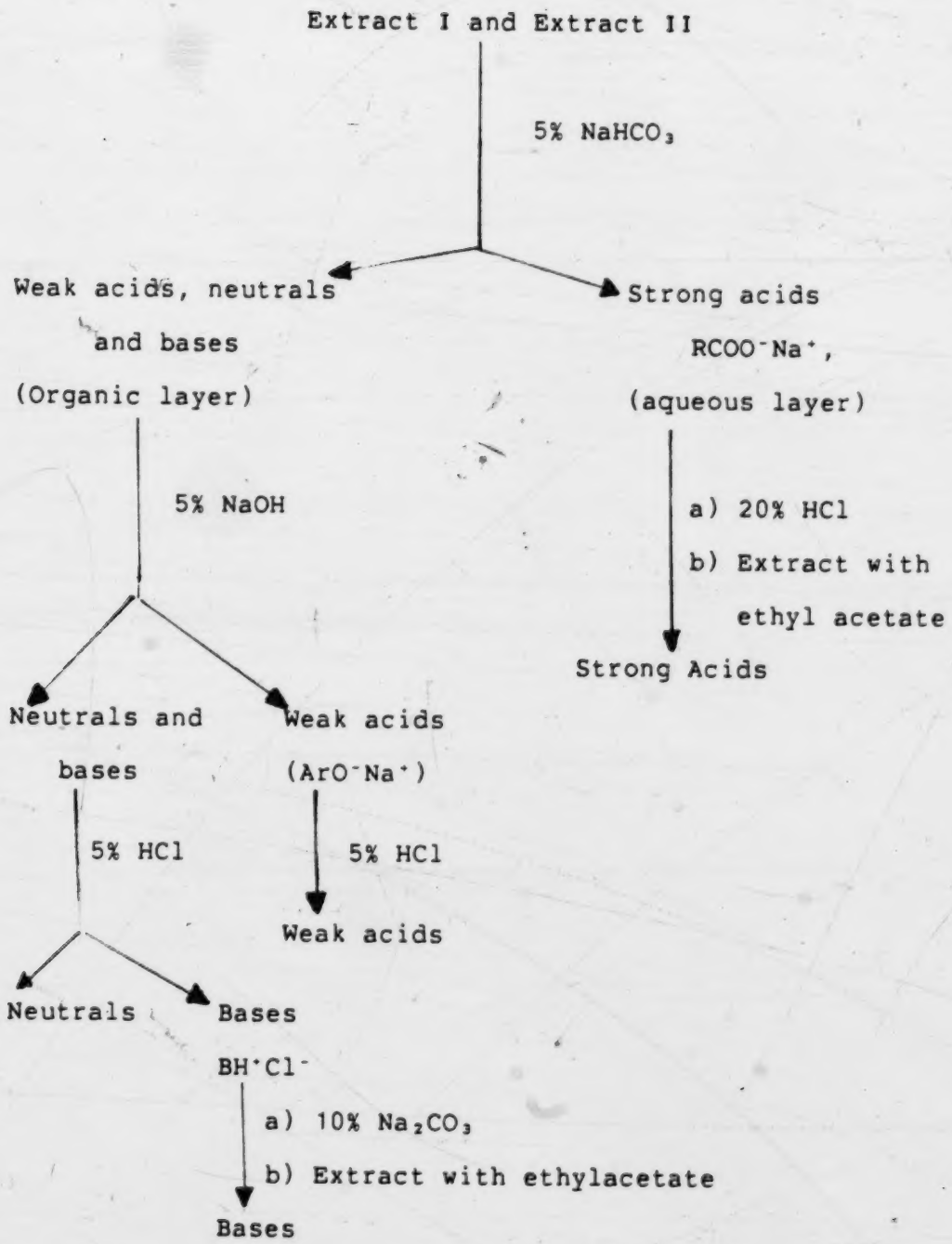


Scheme B : Flow chart of the extraction scheme from the sporocarp of *Fomes roseus* (-continued)





Scheme B continued



Scheme C : Flow-chart of acid, base and neutral extraction

2.2.2 Separations from Extract I

The Skellysolve B extract I was examined first because investigations of other species of closely related genera had revealed that the nonpolar extract contained triterpenoids. Chromatographic separations of extract I by column and/or flash chromatography over silica gel were not encouraging. Complex fractions were obtained even after repetitive chromatographic separations. Only component D was isolated during these separation attempts. Accordingly, extract I was separated into acidic (Ia) and neutral (Ib) compounds (Scheme C), then subjected to chromatography. Table 7 lists compounds isolated from the various Skellysolve B extracts.

2.2.3 Isolation and identification of component D

Component D was obtained from extract I of the sporocarp by a laborious procedure (see experimental) employing one standard and two flash chromatographic separation procedures to give 5 mg of pale yellowish oil which appears as a single spot by TLC.

The FTIR of component D shows absorptions at 3010 cm^{-1} (vinyl C-H stretch), 2953-2854 cm^{-1} (saturated C-H stretch), 1745 cm^{-1} (ester carbonyl) and 1660, 1600 cm^{-1} (C=C stretch). The ^1H NMR spectrum (Figure 1) of this material shows a pattern characteristic of unsaturated triglycerides (80), with peaks at δ 5.38(m, 9H) and δ 4.24(2xdd, 4H). One of the nine protons at δ 5.38 corresponds to the C-2

Table 7.

Compounds isolated from Skellysolve B extract (I, Ia and Ia) of the sporocarp of *F. roseus*

Extract Used	Method of Chromatographic separation	Components Isolated
Direct Skellysolve B (I)	Column and flash column	D
Acidic Skellysolve B (Ia)	Column and flash column	E
Neutral Skellysolve B (Ib)	Column, flash and PTLC	F
	Column and flash column	G
	Column and flash column	H

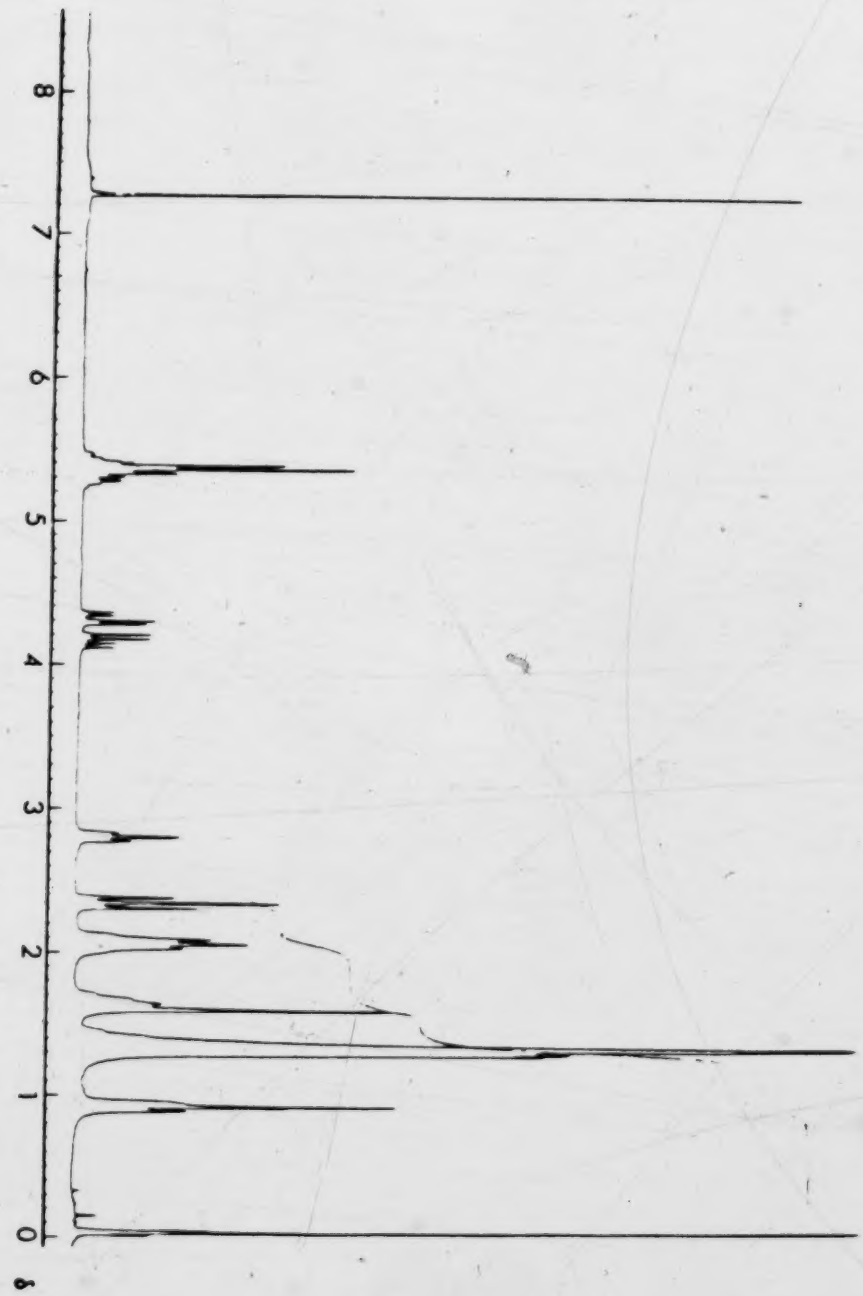
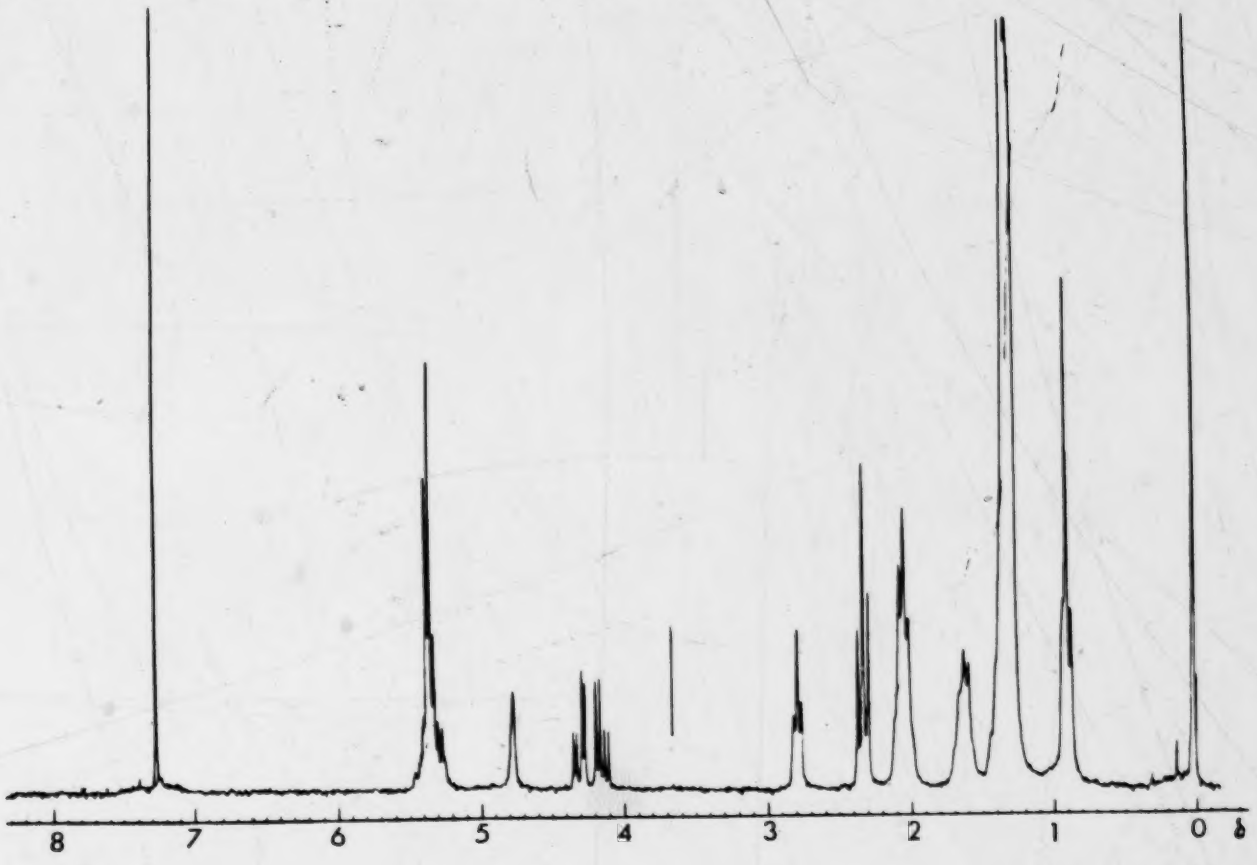


Figure 1 : 200 MHz ^{1}H NMR spectrum ($CDCl_3$) of triglyceride (44).

hydrogen of the glyceride fragment. This proton was shown to be coupled to the protons at C-1 and C-3 of the triglyceride fragment (δ 4.24) by decoupling experiments. The other eight protons at δ 5.38 correspond to olefinic protons of the acyl residues. This was shown by double irradiation experiments since saturation at the doubly allylic and allylic hydrogens of the acyl residues at δ 2.81 and δ 2.06 respectively led to decoupling at δ 5.38. This implies that methylene interrupted unconjugated diene $-CH_2-CH=CH-CH_2-CH=CH-CH_2-$ units are present in the acyl chains. The signals at δ 2.34 and δ 1.66 correspond to the methylene protons α and β to the ester carbonyl respectively. Finally, the signals at δ 1.35 and δ 0.94 correspond to saturated methylenes and terminal methyls respectively. In the 'H NMR of component D there are no D_2O exchangeable protons (Figure 1a). The preceding information defines component D as a triglyceride (44) with acyl residues at the three positions of the glycerol backbone. The fact that the integration for the signals at δ 1.66 corresponds to 8H instead of 6H and δ 2.06 integrates for 10H instead of 8H indicates that the triglyceride is not a single compound. Since no molecular ion was observed from HREIMS, low resolution electron impact mass spectrometry (LREIMS) fast atom bombardment (FAB) and chemical ionization mass spectrometry (CIMS), it is difficult to write the structure for this component. However, four kinds of fragments representing four types of cleavages possible in the molecule, are observed in the

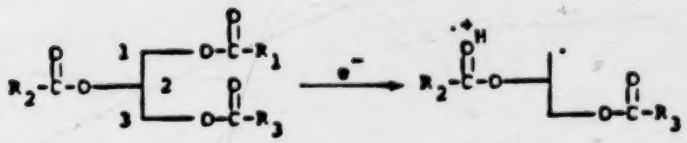
Figure 1a : 200 MHz D,O exchange ^1H NMR spectrum ($CDCl_3$) of triglyceride (44)



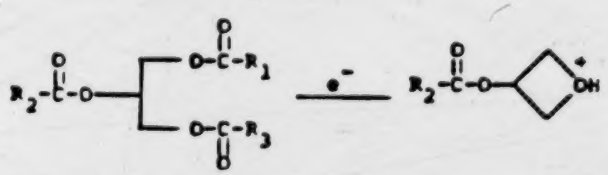
HREIMS.

(1) Fragments containing five oxygen atoms, which represent the loss of an acylium ion from the triglyceride e.g., $C_{30}H_{53}O_5$

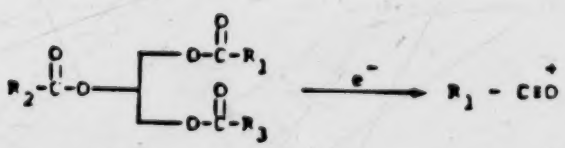
(2) Fragments containing four oxygen atoms, which are formed by loss of a terminal acyloxy fragment from the triglyceride



(3) Fragments containing three oxygen atoms which are formed by the loss of the acyl residues at C-1 and C-3 of the triglyceride e.g., $C_{21}H_{38}O_3$, $C_{21}H_{37}O_3$, $C_{18}H_{37}O_3$.

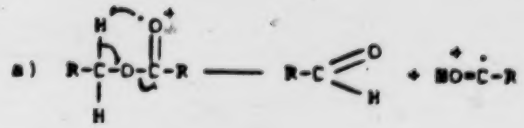


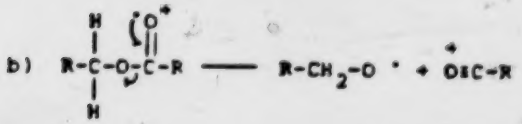
(4) Fragments containing one oxygen atom, which are formed by the cleavage of the ester -CO-O- bond are shown below



e.g., $C_{18}H_{33}O$, $C_{18}H_{30}O$.

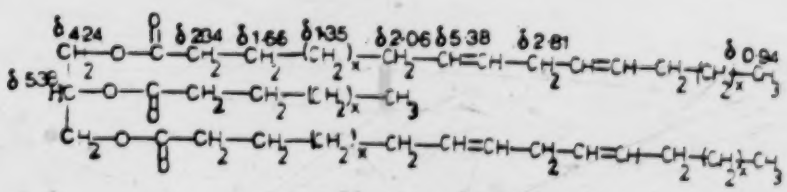
Two fragmentation mechanisms are known to be operative (77). One is favoured at C-1 and C-3 and the other at C-2 of the glyceride backbone.





In conclusion, it is evident that this oil is a mixture of triglycerides which differ in the relative position of the acyl residues and/or by their composition.

A representative structure (44)for the triglycerides can be written and assignment of the 'H NMR spectrum is as follows.



44

Such triglycerides play a vital role in life. They are the major form of energy storage for plants, animals and microorganisms.

2.2.4 Isolation and identification of component E

Component E was isolated form Extract Ia by column and flash column chromatography as a pale yellowish oil and was identical (TLC, FTIR, ^{1}H NMR, HREIMS, etc.,) with linoleic acid (41) isolated earlier from the 4 weeks old malt culture broth.

2.2.5 Isolation and identification of components F, G and H

Extract 1b which was a complex mixture on TLC, was chromatographed over silica gel using gradient elution (Skellysolve B: $CHCl_3$) to give three partially purified fractions. The most abundant fraction 1 (50 mg) was purified by flash chromatography (Skellysolve B: $CHCl_3$) followed by preparative thin layer chromatography (benzene: diethyl ether) to afford component F as a white solid (5mg), mp 160-164°C.

The HREIMS and CIMS results indicate a molecular weight of 396 amu corresponding to the molecular formula $C_{28}H_{44}O$, and the presence of seven unsaturation equivalents. The fragmentation pattern of the molecule is similar to that reported for ergosterol.

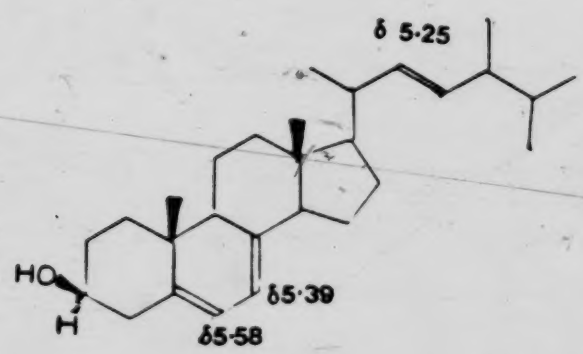
The FTIR shows a broad absorption band at 3400 cm^{-1} characteristic of an intermolecularly hydrogen bonded hydroxyl and olefinic absorptions at 3040, 1660, and 1600 cm^{-1}.

The UV spectrum of component F shows maxima at 262, 271, 282 and 292 nm diagnostic of a conjugated homoannular

diene (81).

The 'H NMR of component **F** is very informative. It shows two quaternary methyl singlets at δ 0.66 and 1.00 and four secondary methyl doublets at δ 0.87(J=7 Hz), 0.89(J=7 Hz), 0.95(J=6.5 Hz) and 1.08(J=6.5 Hz). A carbinolic proton at δ 3.68 (m, 1H, $W_{1/2}$=12 Hz) is characteristic of the 3α proton. Signals at δ 5.58 (dd, J=2.5 and 6 Hz, 1H), 5.39 (ddd, J=2.5, 2.5 and 6 Hz, 1H) and 5.25 (m, 2H) are also shown in the 'H NMR spectrum. These signals are characteristic of olefinic protons. Based on the above spectral information, component **F** was identified as ergosterol (45). The identification was confirmed by comparison with spectra of authentic material.

The 'H NMR signal assignment of some protons of ergosterol (45) is shown on the structure below.



45

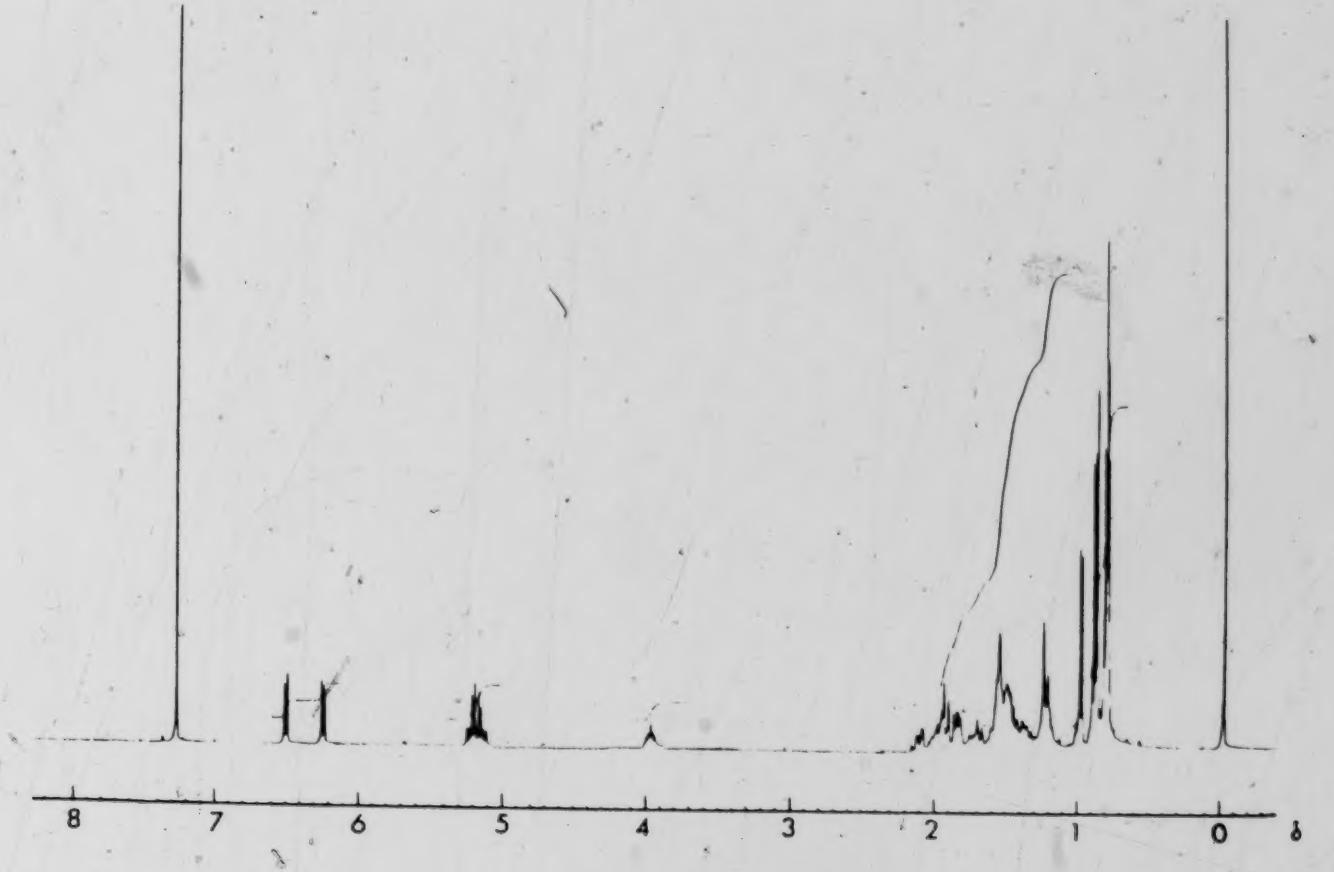
This is a characteristic fungal sterol and has been isolated from various genera of fungi (82).

Purification of fraction 2 (20 mg) by flash chromatography (benzene: diethyl ether) gave component G as white crystals (3 mg), mp 170-174°C.

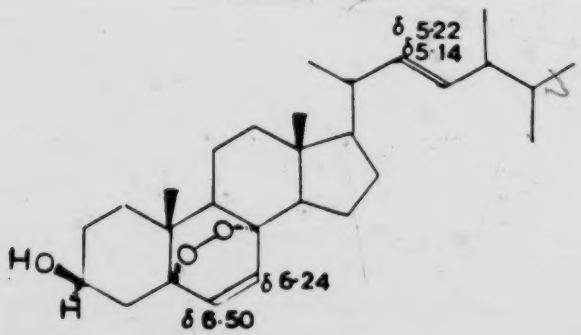
The HREIMS of component G shows a parent ion at m/z 428 corresponding to the molecular formula $C_{28}H_{44}O_3$, and the presence of seven unsaturation equivalents. Loss of oxygen from the molecular ion gives rise to the most abundant fragment ($C_{28}H_{44}O$), a fragmentation that is characteristic of an endoperoxide. The remaining fragmentation pattern follows that of ergosterol (44). This suggests that component G is closely related to component F.

The FTIR of component G shows the presence of a broad hydroxyl absorption at 3400 cm^{-1} (associated OH). The ^1H NMR of component G (Figure 2) is very informative. It shows the presence of two singlet methyls at δ 0.83 and 0.89 and four methyl doublets at δ 0.84 (J=7 Hz), 0.86 (J=7 Hz), 0.91 (J=6.5 Hz) and 0.99 (J=6.5 Hz). An axial carbinolic proton at δ 3.97 appears as a multiplet ($W_{1/2}$=14 Hz). Furthermore, two *vicinal* olefinic protons seen as a doublet of doublets at δ 5.22 (J=7,16 Hz) and 5.14 (J=6.2,16 Hz) indicate the presence of a *trans* substituted double bond. Two other olefinic protons appear as a doublet of doublets at δ 6.24 and 6.50 with a coupling constant of 8 Hz. Based on these spectral properties, component G was identified as ergosterol peroxide (46). This was confirmed by comparison of spectra of 46 with spectra of authentic ergosterol peroxide. The ^1H NMR signal assignment of some protons of

Figure 2 : 200 MHz ^1H NMR spectrum ($CDCl_3$) of ergosterol peroxide (46)



ergosterol peroxide (46) is shown below.



46

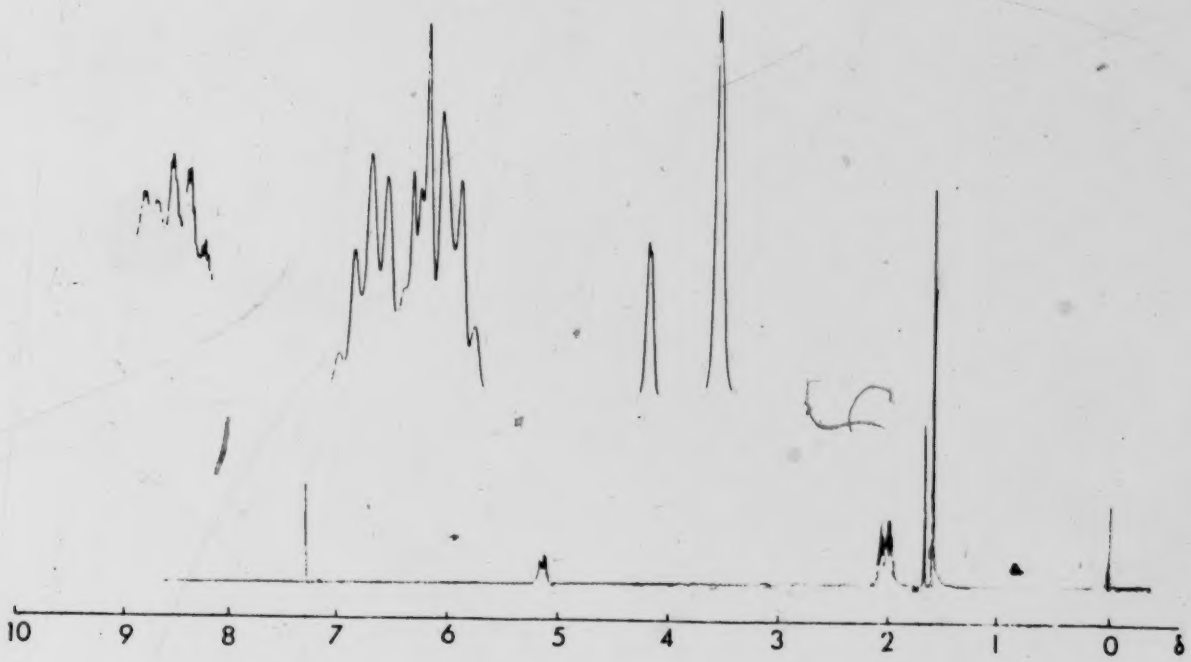
Ergosterol peroxide (46) is a known fungal metabolite which until recently has not been considered biologically active (83). It has been shown to be effective against hepatoma tissue culture (HTC), Zajdela hepatoma cells, an infections ascite cell strain, and normal 3T3 cells.

A low polarity fraction (20 mg) from column chromatography of extract 1b was subjected to further purification by flash chromatography (Skellysolve B: $CHCl_3$). Component H (10 mg) was isolated as a pale yellowish oil which streaks on TLC. The HREIMS of component H shows a molecular weight of 410 corresponding to $C_{30}H_{50}$, and the presence of six unsaturation equivalents are thereby indicated.

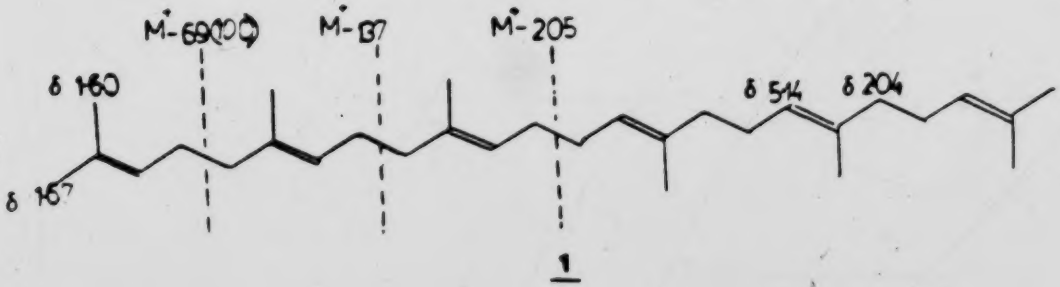
The FTIR of H shows olefinic absorption (3057, 1667 cm^{-1}) and saturated C-H stretch (2965-2854 cm^{-1}).

The ^1H NMR of component H (Figure 3) displays a complex multiplet at δ 5.14 for vinylic protons, a broad multiplet at δ 2.04 for allylic protons and vinylic methyls at δ 1.67

Figure 3 : 200 MHz ^{1}H NMR spectrum ($CDCl_3$) of squalene (1)



and 1.60. Based on the above spectral information, component H was thought to be squalene (1). Comparison of the spectral properties of component H with that of an authentic sample confirmed its identity. The 'H NMR signal assignment and MS fragmentation of 1 are shown below.



1

2.2.6 Separation of Extract II

Extract II is a complex mixture (at least 25 components by TLC) which could not be simplified after repetitive chromatographic separations. The extract was separated into acidic (extract IIw + IIs) and neutral (extract IIb) components in the usual way. Attempted separation of extracts (IIw + IIs) by silica gel chromatography was unsuccessful. Esterification of extract IIb with diazomethane in ether gave extract IIe which gave several fractions upon gradient slow column chromatography (silica gel: eluant 1-5% CH_3OH in $CHCl_3$). One such fraction was purified to give component J (5 mg) as a white solid, mp > 300°C. Component J is a terpenoid of molecular formula $C_{35}H_{50}O_7$ whose spectral and some chemical properties are described in the experimental. Due to the small quantity of

the sample, component J was not investigated further.

2.2.7 Separation of components K and L from extract IIb

Extract IIb contains a mixture of as many as 14 components as revealed by TLC analysis. It was found that slow column chromatography of extract IIb was much more efficient as a separation technique than flash chromatography. Standard column chromatography (silica gel) was carried out according to the elution scheme in Table 8. In this way relatively pure component **K** (8mg) and a less complex mixture (3.5mg) containing component **L** was obtained. Further purification of component **L** from the mixture was achieved by chromatotron centrifugal chromatography (5% CH_3OH in $CHCl_3$) to give component **L** (1.5mg) as a white solid. In an improved and revised elution scheme (Table 9) Component **K** (20mg) and a mixture containing component **K** and **L** (15mg) was obtained. Component **K** (7mg) and component **L** (5mg) were easily obtained on purification of the mixture by PTLC (multiple elution, 5% CH_3OH in $CHCl_3$). Component **K** is one of the major medium polarity components, while component **L** is one of the minor more polar components of Extract IIb.

2.2.8 Identification of Component K as Polyporenic acid-C (47)

Component **K** is a white solid melting between 268-272°C (colour change occurring at 245-250°C). The compound shows

Table 8

Elution profile for the isolation of component K and component L from Extract IIb (1.5g)

Solvent	Volume (mL)	Component isolated
Skellysolve B (Sk.B)	750	Complex mixture
Sk.B: benzene (2:1 & 1:1)	750 each	Complex mixture
Benzene	500	Complex mixture
Benzene: chloroform (2:1 & 1:1)	750 each	Complex mixture
Chloroform	750	Complex mixture
1% Methanol in chloroform	750	Complex mixture
2 and 3% Methanol in chloroform	750	Component K
4% Methanol in chloroform	500	Component K and L
5% Methanol in chloroform	950	Complex mixture
7.5,10,15,20,25% Methanol in chloroform	500 each	Complex mixture
		Complex mixture
Methanol	500	Complex mixture

The polarity and volume of the solvents used was decided on the basis of the visual development of the fractions on TLC.

Table 9

Revised and improved elution scheme for the separation of component K and L from Extract IIb (2.0g)

Solvent	Volume (mL)	Component isolated
Chloroform	1000	Complex mixture
1% Methanol in chloroform	750	Mixture
2% Methanol in chloroform	1000	Component K
3% Methanol in chloroform	2000	Component K
4% Methanol in chloroform	500	Component K and L
5% Methanol in chloroform	1000	Component K and L
Methanol	1500	Complex mixture

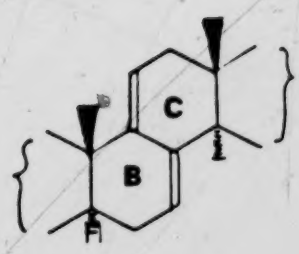
weak activity towards *Staphylococcus aureus*, *S. epidermidis* and *Candida albicans*. It is a highly stable, UV active molecule and is unaffected when left at room temperature for prolonged periods of time. It does not streak on TLC as is characteristic of most carboxylic acids.

Component **K** has been isolated from the neutral fraction extract **IIb**. It shows optical activity $[\alpha]_D^{25} = +13.75$ ($c=0.0008$ g/mL, $CHCl_3$). The biological activity together with its structural complexity makes component **K** the most interesting metabolite isolated from the sporocarp of *Fomes roseus*.

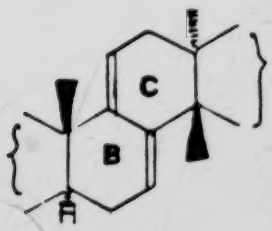
The HREIMS of component **K** indicates a parent peak at 482 amu corresponding to a molecular formula of $C_{31}H_{46}O_4$ and the presence of nine unsaturation equivalents. This weak ion was confirmed as a molecular ion by CIMS using ammonia as a carrier gas. The C_{31} molecular formula suggests that component **K** may be triterpenoid in nature. Furthermore, wood rotting fungi belonging to the family Polyporaceae (Basidiomycetes) are a rich source of triterpenoids (16, 18). The fragmentation pattern in the HREIMS of component **K** shows the absence of dominant fragments arising from the cleavage of ring bonds which are characteristic of pentacyclic triterpenoids. That component **K** may be a tetracyclic triterpene is suggested by the HREIMS fragmentation pattern in which the loss of the side chain ($C_9H_{15}O_2$, 327 amu) and characteristic ring D cleavage fragments ($C_{19}H_{26}O$, 270 amu, which includes loss of a methyl

as well) are observed. Further support for the tetracyclic nature of component **K** arises from the ^{13}C NMR spectrum which shows 3 pairs of olefinic carbons at δ 155.4(s), 144.3(s), 142.2(s), 120.5(d), 117.2(d), and 106.7(t) and two carbonyl carbon resonances at δ 217.6(s) and 178.7(s). This accounts for 5 of the 9 unsaturations implied by the molecular formula. As all the unsaturated functional groups are firmly established, component **K** must contain four rings.

Tetracyclic triterpenes are known to possess the dammarane (3), tirucallane (4), euphane (5), lanostane (6) cucurbitane (7), or fusidane (8) skeletons. The UV spectrum of component **K** shows λmax (CH_3OH) at 237 (log ε 4.15), 244 (log ε 4.22), and 252 (log ε 4.05) nm. These values clearly indicate the presence of a heteroannular transoid conjugated diene system. Thus the dammarane (3) and fusidane (8) skeletons are ruled out for component **K** since such structures cannot accomodate a heteroannular transoid diene. Tirucallane (4, euphane (5), lanostane (6) and cucurbitane (7) skeletons are considered to be possible skeletons for component **K**. The possibility of cucurbitane (7) skeleton for component **K** has been ruled out on the basis of ^1H NMR and circular dichroism (CD) data. The amplitude and sign of CD measurement is in agreement with a structure having 5α configuration. The ^1H NMR of component **K** clearly shows five tertiary methyl groups as singlets. Typical UV absorption maxima of lanostane and euphane $\Delta^{7,9(11)}$ partial ring systems (18) are as follows



λmax 236, 243 and 252 nm



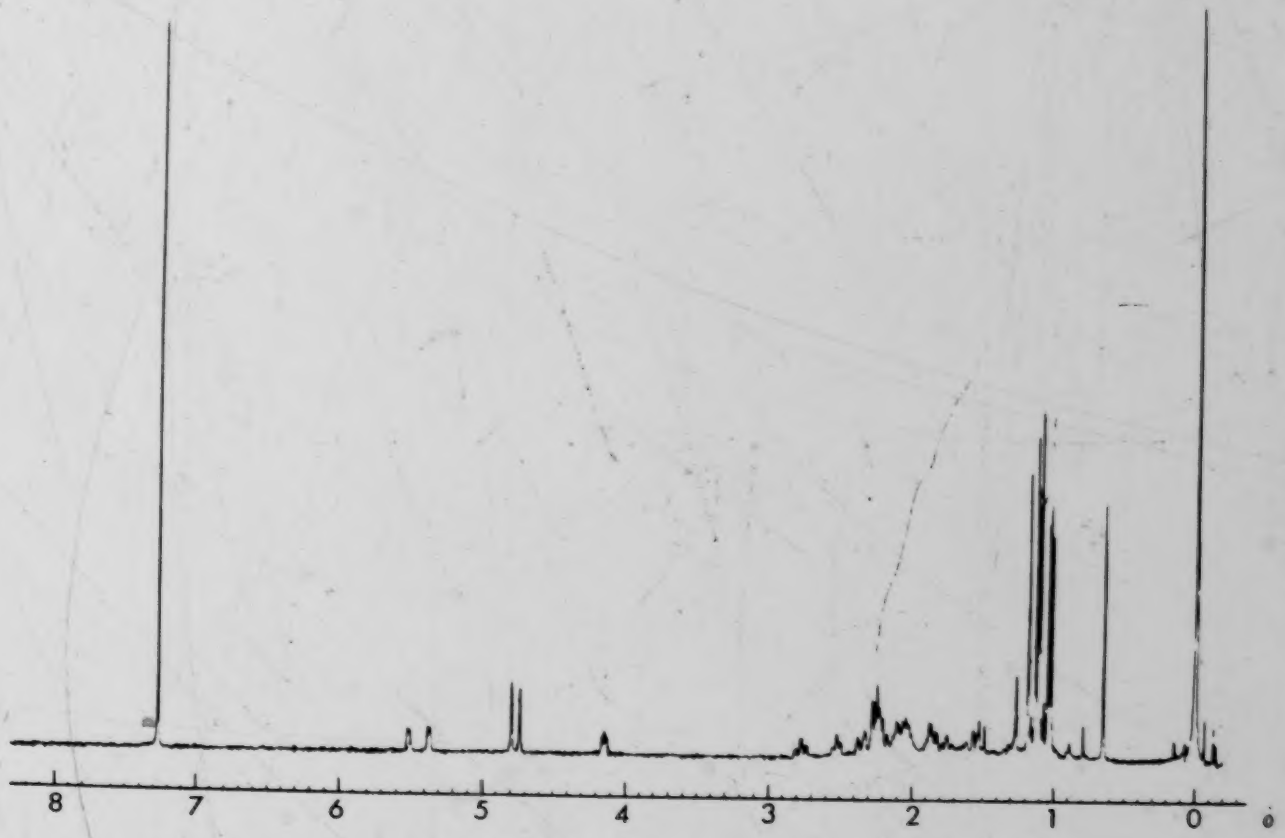
λmax 232, 240 and 247 nm

The UV data shown above clearly rules out the tirucallane (4) and euphane (5) skeletons for component **K** and supports $\Delta^{7,9(11)}$ lanostane ring system.

The FTIR of component **K** shows the presence of a broad band extending from 3600-2400 cm^{-1} with a noticeable peak at 3350 cm^{-1} (associated OH stretching). Moreover, the strong bands at 1714 cm^{-1} and 1680 cm^{-1} confirm the presence of two carbonyl groups in the molecule. The carbonyl absorption at 1714 cm^{-1} suggests a six membered ring ketone functionality, possibly at C-3 in keeping with the fact that the majority of triterpenoids have oxygen functionality at that position. The FTIR absorptions at 1640, 895 and 840 cm^{-1} suggest the presence of an vinylic methylene group and a trisubstituted double bond. Finally, the presence of an isopropyl group was suggested by the presence of peaks at 1380, 1170 and 1138 cm^{-1} in the IR.

The ^1H NMR of component **K** (Figure 4) is very informative. It displays vinylic hydrogens at δ 5.52 (J=7 Hz, 1H) and δ 5.37 (J=7 Hz,1H) as broad doublets. Two broad singlets at δ 4.8(1H) and 4.74(1H) further confirm the

Figure 4 : 400 MHz ^1H NMR spectrum ($CDCl_3$) of polyporenic acid-C (47)



presence of a vinylic methylene group. A carbinyl proton appears at δ 4.15 (ddd, J=1.5, 5.5 and 9 HZ, 1H). The ^{1}H NMR exhibits five tertiary methyl resonances at δ 0.67, 1.10, 1.12, 1.14, and 1.19 (s, 3H each) and two secondary methyl resonances at δ1.01, 1.03 as doublets (J=7 Hz each).

The ^{13}C NMR spectrum of component **K** shows the presence of thirty one carbon atoms in the molecule: 9 quaternary carbon singlets[2] at δ 217.6, 178.7, 155.4, 144.3, 142.2, 48.8, 44.8; 7 tertiary carbon doublets at δ 120.5, 117.2, 76.7, 56.9, 50.7, 46.9 and 34.0; 8 secondary carbon triplets at δ 106.7, 43.3, 36.7, 35.5, 34.9, 32.3, 30.5, and 23.7; and 7 primary carbon quartets at δ 26.0, 25.4, 22.9, 22.5, 22.1, 21.8 and 17.2.

Consideration of the ^{13}C NMR signal multiplicity indicates the presence of 44 hydrogens directly attached to carbons in component **K**. Since this metabolite contains a total of 46 hydrogens (by HREIMS) it is evident that **K** must contain two exchangeable hydrogen atoms. The physical spectral properties mentioned above allow us to speculate that component **K** may be polyporenic acid-C, a fungal metabolite incompletely characterized in earlier reports (54, 84, 85, 86).

In order to verify and assign the structure of component **K** as polyporenic acid-C (47) further analysis of spectral properties and additional experiments were undertaken.

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[2]Multiplicity was determined on the basis of "carppet spectrum".

In the FTIR of component **K** the assignment of the carbonyl at 1680 cm^{-1} to be that of a carboxylic acid rather than an enone was verified by examining the UV spectrum. Acid base shift studies reveal no change in the shape of the UV absorption spectra. This behaviour signifies that the conjugation does not involve a carbonyl functionality and the π-π^* transitions associated with the UV absorption owe their origins to a conjugated diene. That the diene system present in 47 (λmax 237 (ϵ 14125), 244 (ϵ 16595), and 252 (ϵ 10965) nm) is a heteroannular transoid diene is supported by the fact that heteroannular and acyclic dienes have ϵ values between 8000-20,000. Since ϵ values are dependent on the square of the chromophore length, S-*trans* diene maxima are more intense than those of S-*cis*. For example, Component **F** a S-*cis* diene, shows ϵ values of λmax (C_2H_5OH) 262 (ϵ 7200), 271 (ϵ 9260), 282 (ϵ 11800) and 292 (ϵ 6500)nm. The UV spectrum of the derivatives of component 47 show similar λmax values for the three bands in the UV with the central absorption band being more intense than the bands at low or high wavenumber. A closer analysis reveals that the perturbations associated with λmax values due to structural changes in the derivatives of 47 (47a-f, 48, 48a-b) differ by a few nm's with the relative intensity of each of the three bands similar to that of compound 47. A typical UV spectrum of a $\Delta^{7,9(11)}$ diene system is shown in Figure 5. The range of λmax values in the derivatives of 47 and 48 are shown below.

Figure 5 : Typical UV spectrum (CH_3OH) of the $\Delta^{9,(11)}$ diene system.

234-237nm (Intense band)

243-244nm (Most intense band)

251-252nm (Least intense band)

Recently M. Hirotani *et al.* (87) reported the UV spectrum of perennisporiol (50) which has the $\Delta^{7,9(11)}$ lanostane skeleton. In the UV of 50 the intense and least intense bands appear at 232 and 254 nm respectively, while in all other similar compounds the UV values are in agreement with the trend we observe for 47 and its derivatives.

Proof of the position of the ketone in ring A and the stereochemistry at the AB ring junction of 47 is borne out by ORD-CD studies. The ORD shows a plain positive curve as is usually observed with $\Delta^{7,9(11)}$ dienes of the lanostane series. The CD curve shows only one absorption which is the consequence of the asymmetric environment of the carbonyl chromophore of 47. Generally carbonyl compounds exhibit weak UV absorption (n-π^* transition) which permits rotatory dispersion measurements through the region of maximal absorption (88). The Cotton effect typical of the optically active n-π^* transition of the saturated ketone in the 300 nm region is exhibited at $[\phi]_{300} = -483.78°$. The amplitude and sign of the CD of 47 is in agreement with 3-keto-4,4-dimethyl-5α-compounds, i.e., 4,4-dimethylcholesta-3-one, 4,4-dimethyldihydrotestosterone, and 4,4,17α-trimethyldihydrotestosterone (88). A list of Cotton effect parameters (sign and amplitude) of various

partial ring structures are shown in Table 10. Thus ORD-CD, UV data define the stereochemistry at the AB, CD ring junctions and at the C_{17} side chain. This information supports our structural assignment of polyporenic acid-C (47) for component K.

The ^{13}C NMR chemical shift of sp^2 hybridized carbons in the $\Delta^{7,9(11)}$ diene of several lanostanes and compound 47, 47a, 48a etc are listed in Table 11. The ^{13}C values (δ) of $\Delta^{7,9(11)}$ diene moiety of 47 agree closely with other reported compounds (17, 49) as shown in Table 11.

Acetylation and methylation studies of component K conclusively demonstrate the presence of hydroxyl and carboxylic acid groups in the molecule. Esterification of component K with CH_2N_2/ether gives 47a. The IR spectrum of 47a does not show the 3200-2600 cm^{-1} band but absorption due to hydroxyl stretching is observed at higher frequency (3418 cm^{-1}). Acetylation of component K with acetic anhydride and pyridine forms an acetyl derivative 47b, the IR spectrum of which shows that the characteristic acid band (3200-2600) cm^{-1} is retained, while no band at 3350 cm^{-1} is observed. The ^1H NMR of 47a shows methyl ester resonance at δ 3.70 (s 3H) while 47b shows acetyl methyl resonance at δ 2.06 (s, 3H). Thus ^1H NMR and FTIR studies confirm the presence of hydroxyl and carboxylic acid groups in component K.

In order to prove the position and stereochemistry of the hydroxyl group in 47, oxidation, dehydration, acetylation reactions together with a literature search were

Table 10

List of Cotton effect (sign and amplitude) in various partial ring structures

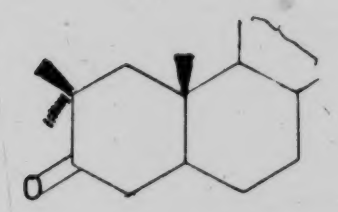
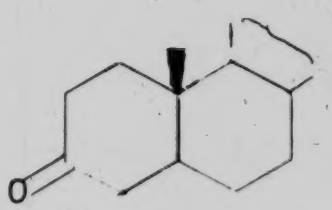
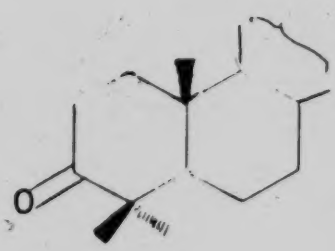
Structure	Sign	Amplitude
	+ve	>1800°
	+ve	>1500-1800°
	-ve	<500°

Table 11

^{13}C NMR spectral data of $\Delta^{7,9}(^{11})$diene compounds

Carbon atom (multiplicity)	17	47	47a	48a	49	50[1]	50a[1]	50b[1]	50c[1]	50d[1]	52	52a
C-7 (d)	120.1	120.5	120.6	121.0	120.8	121.3	121.6	121.1	121.3	121.4	118.0	115.8
C-8 (s)	142.7	142.2	141.9	141.6	141.9	140.1	140.6	140.2	140.7	140.8	135.7	136.3
C-9 (s)	145.9	144.3	144.3	145.7	145.8	145.9	146.3	145.6	146.0	144.9	143.4	140.1
C-11 (d)	116.3	117.2	116.9	115.8	115.9	115.9	116.2	116.2	116.6	117.4	118.8	122.8

[1] Unassigned by the authors (87).

undertaken.

Efforts to oxidize polyporenic acid-C (47) by previously reported procedures using CrO_3/H_2SO_4 at room temperature (89) were not successful. We observed that this oxidation destroyed the heteroannular diene system present in the molecule since at least three products appeared on TLC none of which were visible by ultraviolet irradiation. No attempt was made to analyse the products. This reaction indicates that 47 appears to be acid sensitive and oxidized extensively. Firouzabad *et al.* (90) have recently prepared a reagent known as tetrakis(pyridine)silver dichromate, a stable neutral reagent useful for the oxidation of benzylic and allylic alcohols. We employed this reagent for oxidation of the secondary alcohol in component **K**. Thus oxidation of component **K** (2mg) with excess tetrakis(pyridine)silver dichromate in dry benzene after improved work up gave an oxidation product 47d whose UV spectrum did not show absorptions (225(sh), 290(ϵ 10800) nm) characteristic of a conjugated dienone (81). Thus the hydroxyl group in component **K** is not located at the allylic positions C-6 or C-12. The UV spectrum of the oxidation product 47d, which is similar to that of component **K**, shows an additional weak intensity inflexion at 384(ϵ 78) nm. The FTIR of 47d shows an absorption at 1737 cm^{-1} characteristic of a carbonyl group in a 5-membered ring (91). This value is not in agreement with that reported by Bowers *et al.* (89) for polyporenic acid-C (Table 12). The carbonyl region

Table 12

Comparison of carbonyl region absorption of component K and its derivatives

Compound	Observed data[1]	Data reported by		
		Bower *et al.*	Pinhey *et al.*	Natori *et al.*
47	1714, 1680, 1640	1712, 1639[2]	-	
47a	1727, 1682, 1639	1738, 1712, 1639[2]	1725, 1645	1735, 1710(sh), 1685[3]
47b	1732, 1700, 1640	-	-	
47c	1735, 1709, 1638	-	-	
47d	1737, 1707, 1640	-	-	
47e	1735, 1709, 1640	1743, 1727, 1711, 1639[4]	-	-
47f	1640	-	-	-
47(h+ha)	1735, 1710, 1640	-	-	
47J	1767, 1747, 1707, 1638	1766[5]	-	
48	1736, 1640	-	-	
48a	1732, 1640	-	-	-

[1]CHCl$_3$ cast, [2]CCl_4, [3]KBr, [4]Nujol and [5]From lactonization of 47a

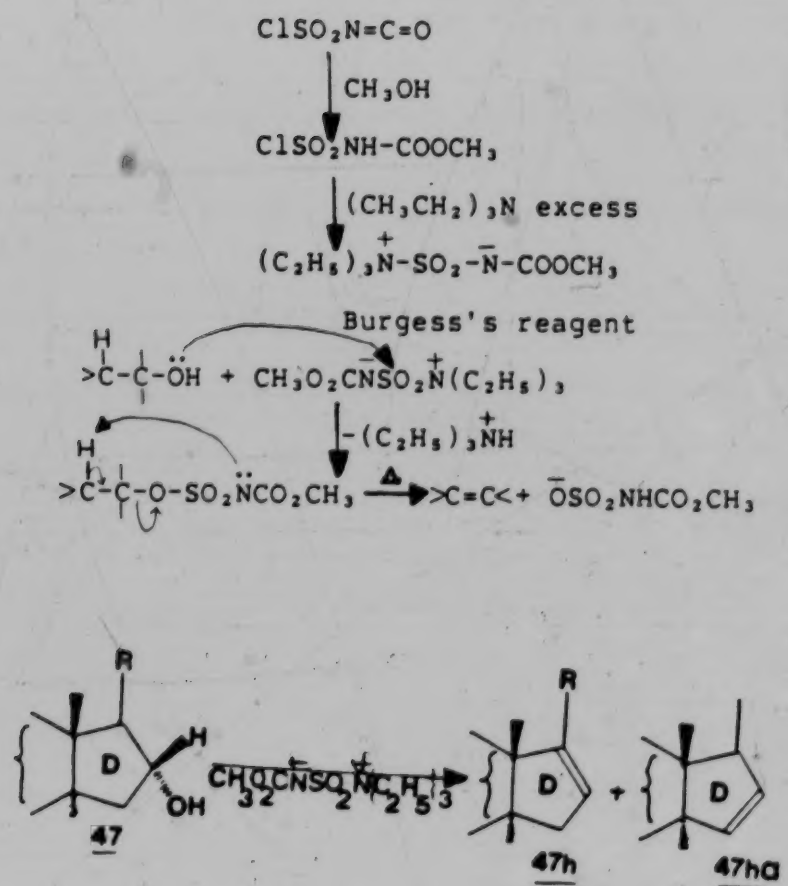
absorptions of 47 and its various derivatives including incomplete data from other workers has been listed in Table 12. The evidence presented shows that in 47 the hydroxyl group must be either at C-15 or C-16.

To confirm the position of hydroxyl group in the 5-membered ring of component K, a dehydration reaction with carboxysulfamoyltriethylammonium hydroxide inner salt methyl ester (Burgess's reagent, $MeO_2CNSO_2N(C_2H_5)_3$) (92) was employed. Treatment of 47 with Burgess's reagent for 8 hours in dry benzene under reflux gave two dehydration products 47h + 47ha in approximately equal amounts. The dehydration products, which were overlapping spots by TLC, could not be separated by regular or argentation chromatography. Furthermore, the dehydration products were not stable. Since two products were apparently formed on dehydration, the hydroxyl function is likely located at C-16.

The sequence of the reactions involved in the preparation of the Burgess's reagent and the course of the dehydration reaction has been rationalized in Scheme D.

The configuration of hydroxyl group in component K as α was deduced from the following literature data as shown in Figure 6.

No examples of a β hydroxyl group at C-15 in the lanostane or euphane series were found. Based upon this information, the course of dehydration reaction to give two products and the shape of the signal at δ 4.15 in the ^1H NMR of 47, the position of hydroxyl is established as 16 α.



Scheme D : Preparation of Burgess's reagent and the course of dehydration of polyporenic acid-C (47)

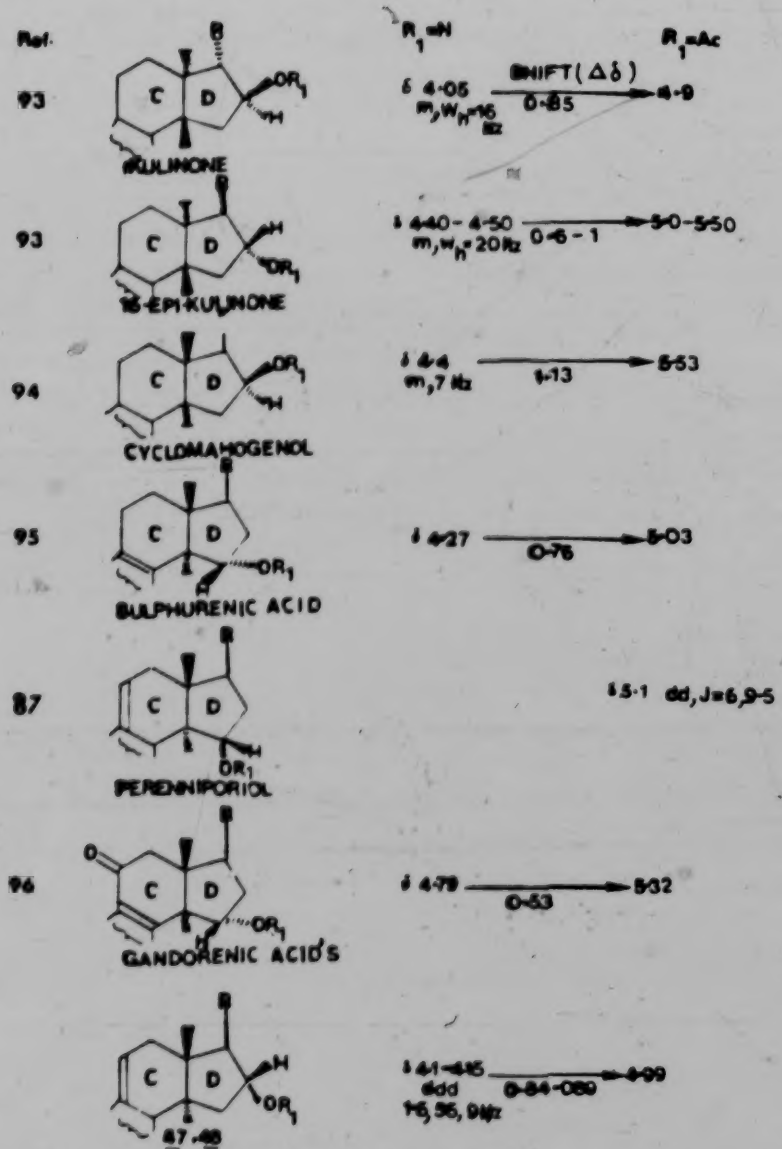


Figure 6 : Carbinyl proton chemical shift in the 5-membered ring of the lanostane and euphane series of tetracyclic triterpenes and their chemical shift(δ) on acetylation.

Further support in favour of α configuration of hydroxyl group in polyporenic acid-C was deduced from the fact that acetylation of 16α-hydroxy steroids results in a large negative shift in the molecular rotation while 16β-hydroxy steroids gives a positive shift (89). This is in agreement with our observed shift in rotation from +10° to -10° while going from 47a to 47b respectively.

In order to establish the position of the acid functionality at C-20 in polyporenic acid-C (47) a lactonization reaction with p-toluenesulphonic acid (PTSA) in benzene was attempted under reflux (water bath, 12 hrs.). The FTIR of the mixture 47J revealed the presence of two bands in the carbonyl region at 1770 cm^{-1} and 1747 cm^{-1}, characteristic of γ and δ lactones respectively. These results indicate that the double bond is δ to the carboxylic acid group and that the OH group is γ to the carboxylic acid group.

These investigations support the structure of component K as polyporenic acid-C (47). This is the first reported isolation of 47 from *Fomes roseus*.

Zellner and his coworkers (97) first examined metabolites produced by the birch tree fungus *Polyporus betulinus* in 1913. Cross and coworkers (98) reinvestigated the birch tree fungus, a large parasitic white shelf fungus and claimed to have isolated an analytically impure sample of polyporenic acid-C, mp 263-266°C. They reported that polyporenic acid gave an orange coloured lower layer with

$CHCl_3$ and H_2SO_4, while it gave a blue-violet colouration with acetic anhydride and sulphuric acid. The molecular formula $C_{30}H_{48}O_4$ or $C_{30}H_{48}O_4$ was suggested on the basis of the analysis of methyl polyporenate, mp 192-193°C. Because of the complexity of the data, further investigation of the molecule was abandoned.

Birkinshaw, Morgan and Findlay (99) grew *P. benzoinus* on a synthetic medium for 10 weeks. The wet mycelium was removed and minced with ether, the solvent was decanted, dried and concentrated to yield a viscous fawn colored paste. Treatment of the paste with ether gave a white insoluble portion which was isolated and identified as polyporenic acid-C. The acid gave color reactions characteristic of triterpenoid acids. The behaviour of the acid on melting is characteristic: at 250°C, it begins to discolor; at 275-280°C it begins to soften; at 285-290°C it melts with liberation of gas. It has a rotation $[\alpha]_{5461}^{20}$= 8.91(c=5.1 pyridine). The methyl ester has a rotation of $[\alpha]_D^{21}$= 19.5°(c=6.7, chloroform). Polyporenic acid-C was reported to form a diacetyl derivative [mp 212-214° decomp, $[\alpha]_D^{21}$= +3.2 (c=8.6, chloroform)]. The very fact that a diacetyl derivative was reported shows that these workers were dealing with impure material. Our studies have conclusively established that polyporenic acid-C is a monohydroxy acid and not a dihydroxy acid.

Bowers, Halsall, Jones and Lemin (89) isolated an acid from *Polyporus benzoinus* and named it benzoinus acid. The IR

of the methyl ester of this acid was surprisingly reported (without quoting IR absorptions) to be similar to the methyl ester of the dihydroxy acid i.e., the so-called polyporenic acid-C reported by Birkinshaw *et al.* (99). Bowers reported that isolation of appreciable quantities of polyporenic acid-C was difficult due to the lack of a suitable method for estimating purity. This handicap was overcome when methyl polyporenate-C was shown to contain a conjugated diene system and a keto group. Bowers *et al.* (89) established the structure with the configuration of the hydroxyl group at C-16 as β, while Halsall and Sayer (100) showed the configuration of this hydroxyl group as α.

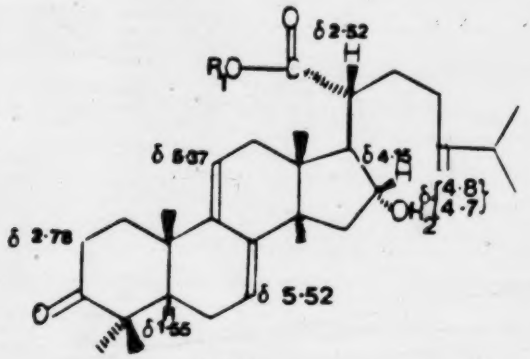
All previous workers (54, 84, 85, 86, 89) have reported great difficulty in isolating polyporenic acid-C. Their data is incomplete and often conflicting perhaps due to impurities of other closely related inseparable compunds. Polyporenic acid-C has been reported by Natori *et al.* (16) from various sources:
Daedalea dickinsii, D. tanakae, Fomitopis pinicola, Melanoporia cajanderi, M. Juniperina, M. purpuracea, M. nigra, M. rosea, Piptoporus betulinus, Tramates dickinsii, T. feei, and Poria cocos. However, there is no reliable detailed ^{1}H NMR, ^{13}C NMR and mass spectral data for 47 and its derivatives.

The isolation of polyporenic acid-C from *Fomes roseus* has allowed us to clarify the literature values and this work leads to the assignment of its structure as 47 with

certainty.

2.2.9 Assignment of 'H NMR signals

The 'H NMR signal assignment of polyporenic acid-C (47) and some of its derivatives is discussed below.



47

R_1=H, R_2=H polyporenic acid-C (47)

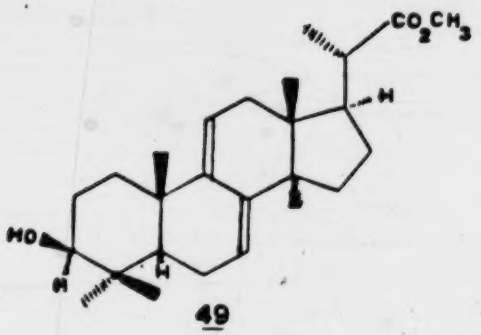
R_1=CH_3, R_2=H methyl polyporenate-C (47a)

R_1=H, R_2=$COCH_3$ O-acetylpolyporenic acid-C (47b)

R_1=CH_3, R_2=$COCH_3$ methyl O-acetyl polyporenate-C (47c)

The vinylic hydrogen H-7 appears as a broad doublet at δ 5.52 and is coupled to another broad doublet at δ 5.37 (H-11) as shown by decoupling experiments. Irradiation of H-7 changes the signal at δ 1.8-2.3 and this is assigned to the *geminal* protons at C-6 (AB Portion of an ABMX Spin system). The appearance of H-7 as a broad doublet (X part of an ABMX spin system) is slightly altered by irradiation of the well resolved double doublet at δ1.55 (M part of an ABMX spin system, J=3.5 and 11 Hz) assigned to H-5. The configuration of H-5 is axial (α) since it shows one large

and one small coupling (J(5ax-6ax)=11 Hz, J(5ax-6eq)=3.5 Hz). Irradiation of H-11, X part of an ABX spin system causes decoupling at δ 2.43 and 2.17 and these signals are assigned to the C-12 protons. The assignment of the vinylic hydrogens (H-7, H-11) in the 'H NMR of polyporenic acid-C (47) is in agreement with the chemical shifts reported for the vinyl protons of compound 49), a metabolite of the entomopathogenic fungus *Verticillium lecanii* (101).

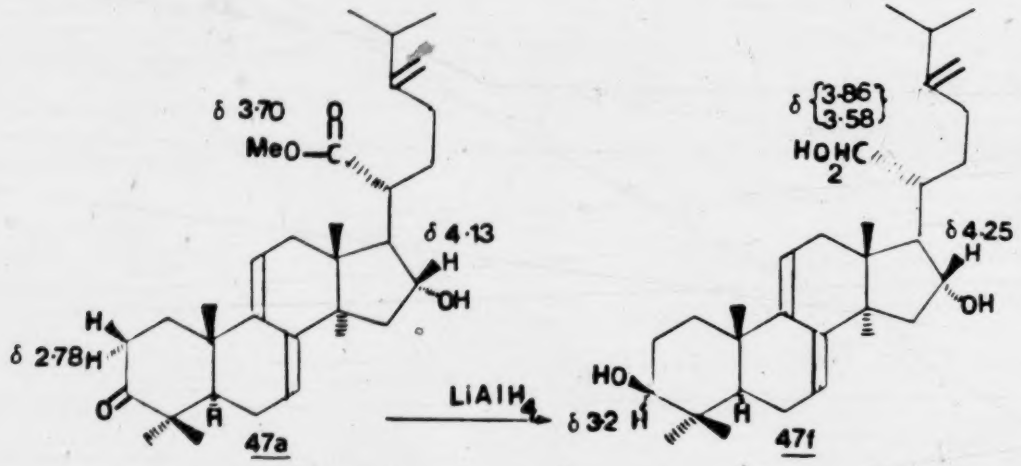


49

In the 'H NMR of 47 the vinylic methylene protons at C-28 appear as two broad singlets at δ 4.8 and δ 4.74. Interestingly, in the 'H NMR of 16-O-acetylpolyporenic acid (47b) the vinylic methylene protons appear as doublets and are shifted upfield to δ 4.76 (J=1 Hz) and 4.63 (J=1 Hz). The signal at δ 4.15 (ddd, J=1.5, 5.5 and 9 Hz, 1H) in the 'H NMR of 47 is shifted downfield in the 'H NMR of 47b to δ 4.99 and these signals are assigned to H-16 α as discussed earlier.

The signal at δ 2.78 (ddd, J=6, 14 and 14 Hz, 1H) in the 'H NMR of methyl polyporenate-C (47a) is assigned to an

axial proton (H-2), α to the ring A carbonyl group on the basis of following experimental evidence. Upon reduction of (47a with LiALH, a triol 47f is formed. In the 'H NMR of 47f (Figure 7) the signal at δ2.78 is not observed. Furthermore, treatment of either polyporenic acid-C (47) or methyl polyporenate (47a) with NaBH, led to the formation of the reduction products 48 and 48a, respectively. In the 'H NMR of 48 or 48a no signal at δ 2.78 (H-2 axial in 47) is observed. In each case an axial carbinyl proton as a double doublet (J=4, 11 Hz, 1H) at δ3.2 is observed. It is interesting to note that double doublets due to diasterotopic hydrogens at C-21 (δ 3.86, J=2, 11 Hz, 1H) and (δ 3.58, J=2.5 and 11 Hz, 1H) J *geminal*=11 Hz appear in the 'H NMR of triol 47f.



The signal at δ 2.52 (dt, J=3.0, 11 Hz, 1H) in the 'H NMR spectrum of methyl polyporenate-C (47a) is assigned to H-20 (the proton which is α to the COOH group in the side chain) on the basis of following spectral information. This

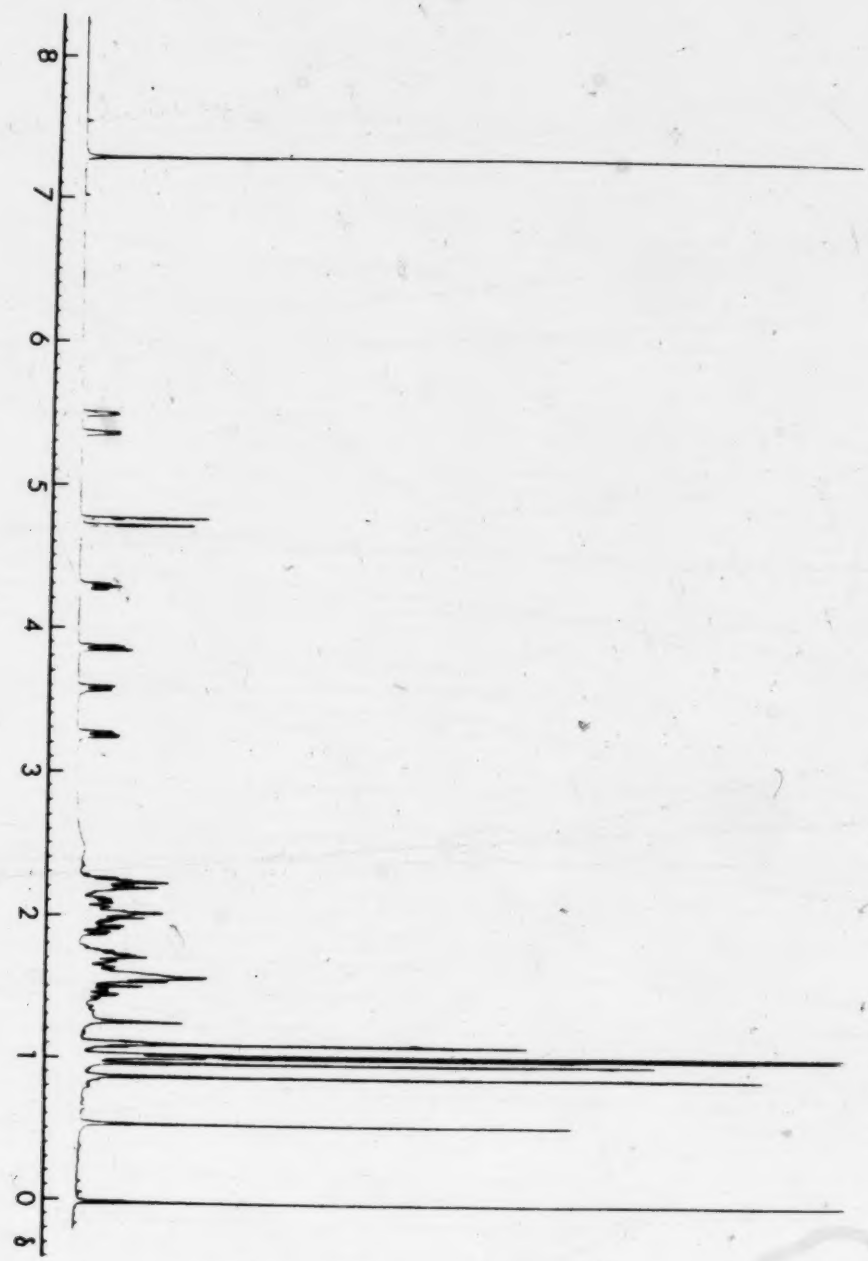
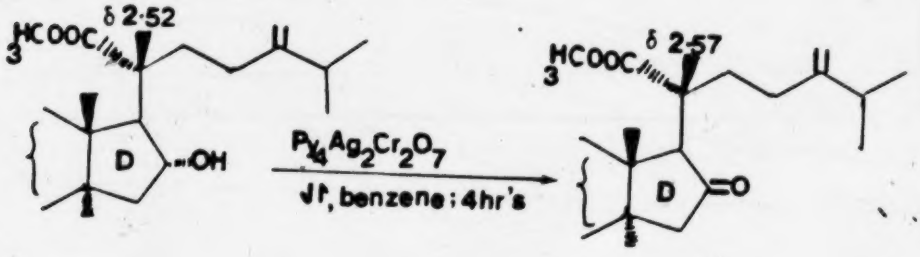


Figure 7 : 400 MHz ^{1}H NMR spectrum ($CDCl_3$) of triol 47f

signal is no longer discernible in the 'H NMR of the reduction product, triol (47f). Moreover, the carbinyl proton (H-16 α) is shifted downfield by 0.12 ppm in the 'H NMR of the triol 47f relative to the 'H NMR of methyl polyporenate-C (47a).

Conversion of methyl polyporenate-C (47a) to its 16-oxo derivative (47e) was accomplished using tetrakis(pyridine) silver dichromate. In the 'H NMR of 47e (Figure 8), H-20 is observed at δ 2.57, shifted downfield by 0.05 ppm with respect to the chemical shift observed in the 'H NMR of 47a. This small downfield shift suggests that the proton is in close proximity to the C-16 carbonyl.

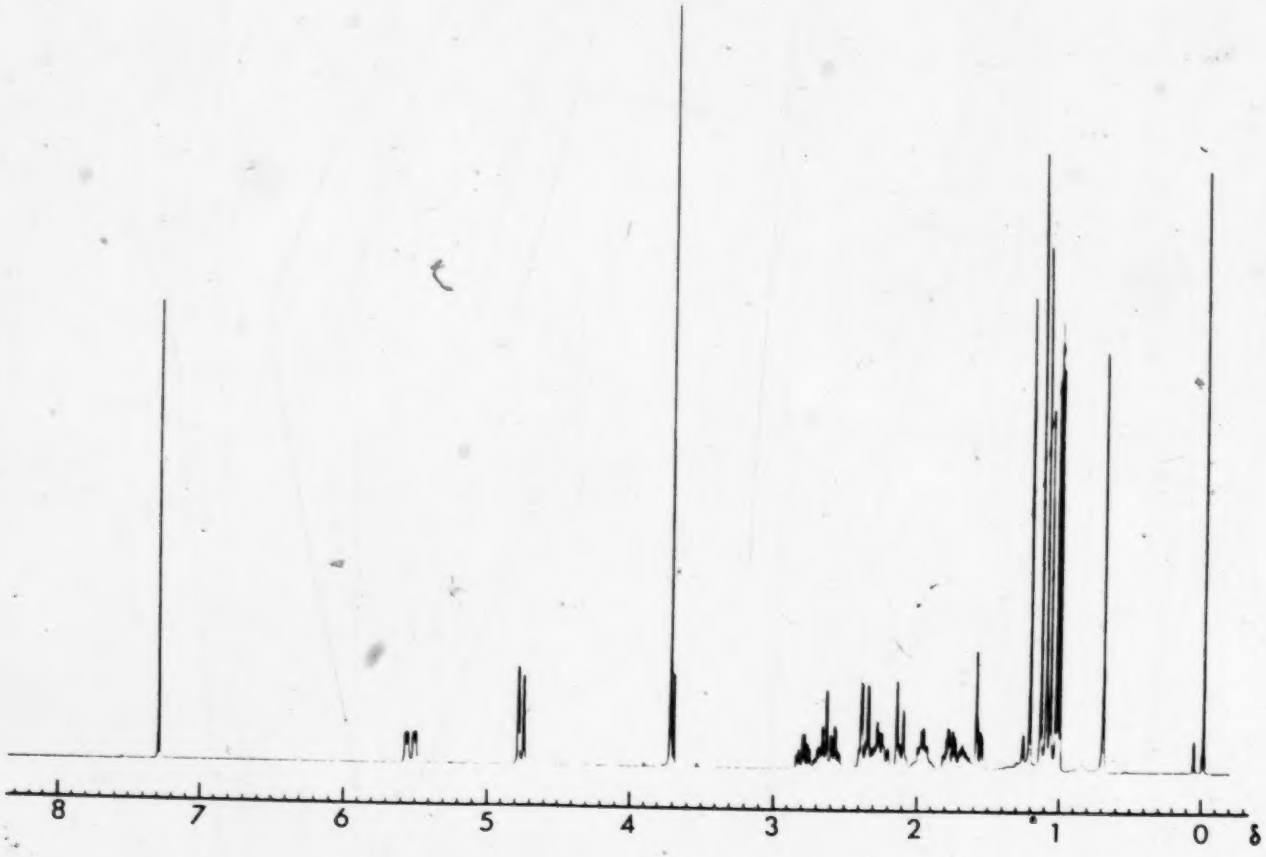


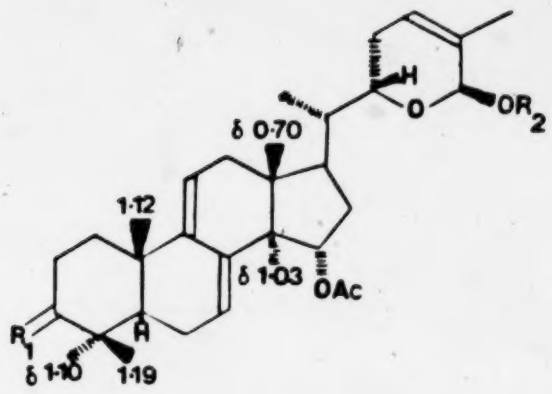
Partial structure of ester 47a

Partial structure of 16-oxo ester 47e

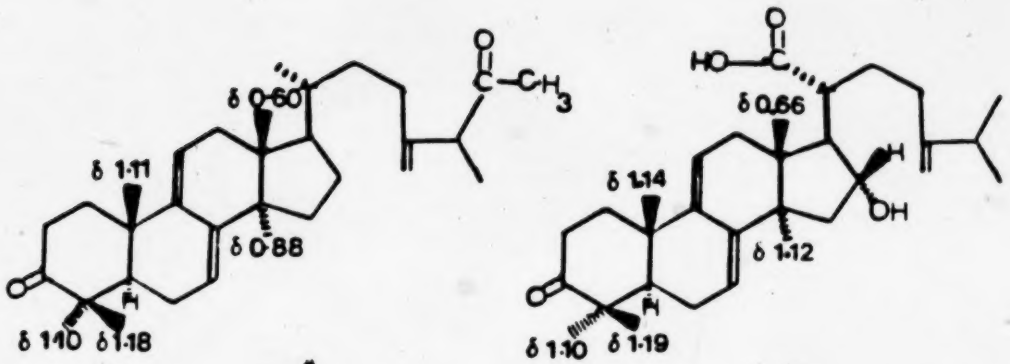
The angular methyl resonances of several lanostane compounds have been compared in Figure 9. In our assignment of angular methyls for tyromycic acid (51), the C-32 methyl group must resonate at δ 0.88 while in 26-O-methyl-3-oxo-perreniporiol (50), this methyl resonance has been assigned at δ 1.03 (87). We assigned the C-32

Figure 8 : 400 MHz ^1H NMR spectrum ($CDCl_3$) of methyl 16-oxo-polyporenate-C (**47e**)





50	R_1 = OH, H	R_2 = Me
50a	R_1 = OH, H	R_2 = H
50b	R_1 = OAc, H	R_2 = Me
50c	R_1 = OAc, H	R_2 = H
50d	R_1 = O	R_2 = Me



51* 47*

Figure 9 : Comparison of angular methyl resonances of various $\Delta^{7,9(11)}$ lanostane compounds (47, 50, 51): * our assignment

methyl resonance of polyporenic acid-C (47) to be that observed at δ1.12 since the pseudo-1,3-diaxial relationship with the C-16 hydroxyl group would be expected to induce a downfield shift. Y.Kawazoe *et al.* (102) have reported the ^{1}H NMR chemical shift data for a series of alcohols and their corresponding acetate derivatives. Acetylation of 47 gave O-acetylpolyporenic acid-C (47b) whose ^{1}H NMR spectrum shows the C-32 methyl group at δ1.05, shifted downfield in accordance with the observation of Kawazoe.

The ^{1}H NMR methyl resonances of polyporenic acid-C (47) and its derivatives are listed in Table 13.

2.2.10 ^{13}C NMR assignment in polyporenic acid-C (47) and methyl polyporenate-C (47a)

The assignment of the part of the side chain from C-24 through C-28 in (47, 47a was made by direct comparison (103, 104) with the spectral data for cycloeucalenol acetate (53) and $\Delta^{24(28)}$ methylene cholesterol (54)as shown in Figure 10. The assignment of signals at C-20 and C-22 has been made on the basis of observed multiplicities of the respective carbon atoms aided by the process of elimination. The ^{13}C resonance values (105) of methylene cycloalkanes (Figure 11) substantiates that vinylic methylene in polyporenic acid (47) etc., is not the part of the nuclear framework. The assignment of the C-16 carbinol carbon at δ 76.7 was also been facilitated by comparing the ^{13}C data (106) of hydroxy steroids (Figure 11). Finally the assignment of ^{13}C

Table 13

^{1}H NMR methyl resonances of polyporenic acid-C (47) and its derivatives

Compound	C-18CH$_3$,	C-19CH$_3$,	C-26CH$_3$, and C-27CH$_3$, (J=7 Hz)		C-30CH$_3$, (4α)	C-31CH$_3$, (4β)	C-32CH$_3$,
47	0.66, s	1.14, s	1.01, d	1.03, d	1.10, s	1.19, s	1.12, s
47a	0.62, s	1.14, s	1.01, d	1.03, d	1.09, s	1.18, s	1.12, s
47b	0.69, s	1.13, s	1.00, d	1.02, d	1.10, s	1.19, s	1.05, s
47c	0.64, s	1.12, s	1.00, d	1.03, d	1.10, s	1.19, s	1.05, s
47e	0.71, s	1.15, s	1.00, d	1.02, d	1.11, s	1.22, s	1.07, s
47f	0.55, s	0.97, s	1.00, d	1.02, d	0.88, s	1.03, s	1.10, s
48	0.62, s	1.04, s	1.00, d	1.02, d	0.89, s	0.97, s	1.11, s
48a	0.60, s	1.03, s	1.00, d	1.02, d	0.89, s	0.97, s	1.10, s

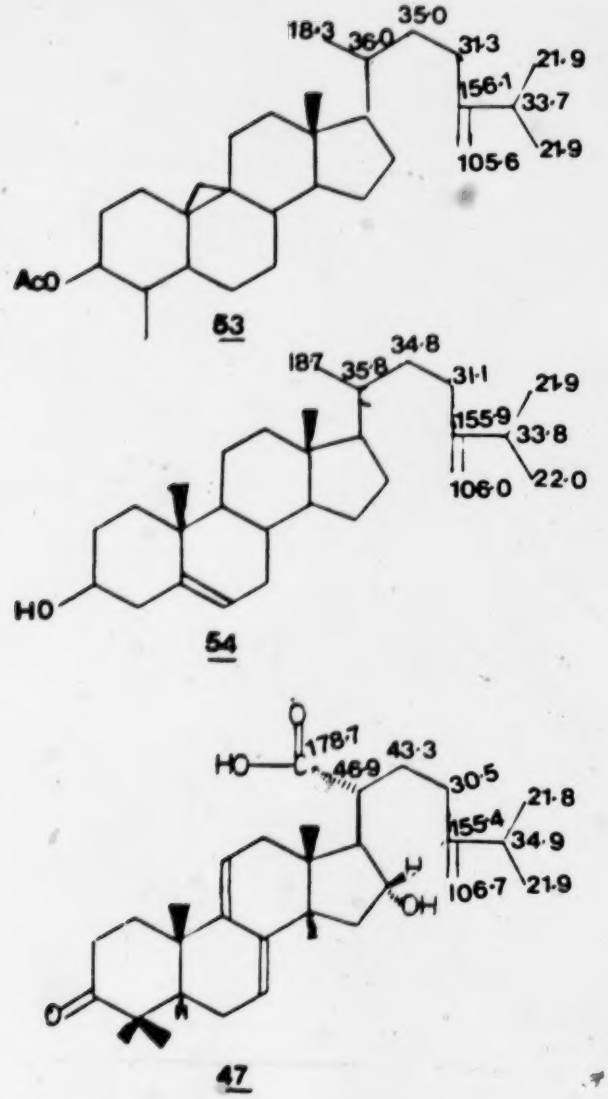
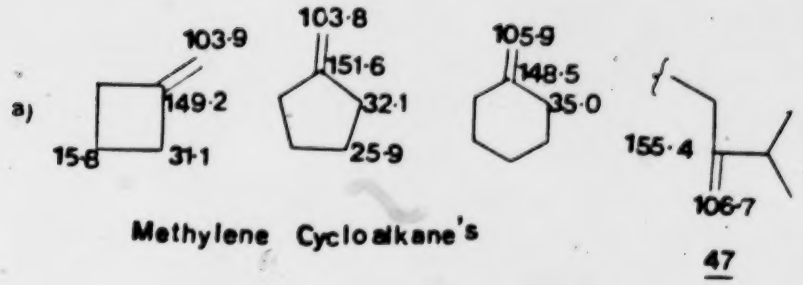
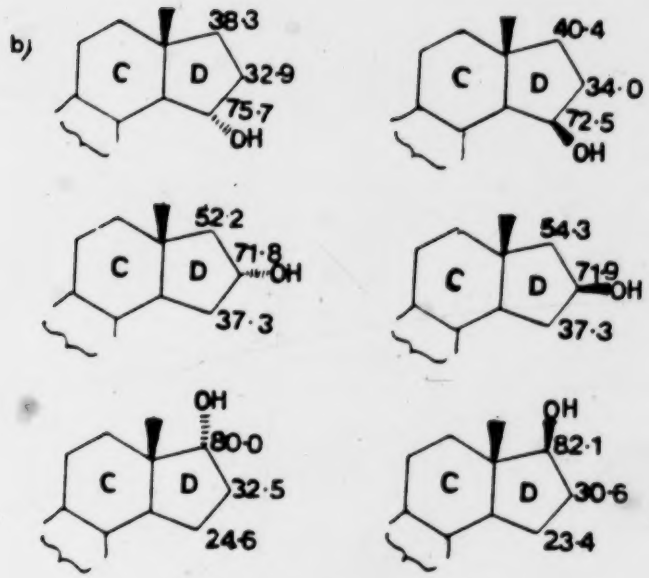


Figure 10 : Comparison of the side chain ^{13}C NMR spectral data of cycloeucalenol acetate (53), $\Delta^{24(28)}$ cholesterol (54) and polyporenic acid-C (47)

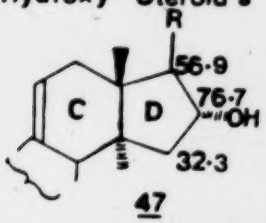


Methylene Cycloalkane's

47



Hydroxy Steroid's



47

Figure 11 : a) Comparison of ^{13}C NMR spectral data of vinylic methylene compounds,

b) Comparison of ^{13}C NMR spectral data of hydroxy steroids and polyporenic acid-C (47)

resonances in the nuclear frame work of 47 and 47a was aided by comparison with reported ^{13}C literature values (101, 107) for agnosterol (lanosta-7,9(11)-dien-3β-ol, 17a) and 3-β-hydroxy-4,4,14αtrimethyl-5α-pregna-7,9(11)-diene-20 (S)-carboxylic ester (49) and gandoreic acids (96).

The ^{13}C resonances in polyporenic acid-C (47) and methyl polyporenate-C (47a) are shown in Figure 12.

2.2.11 The mass spectral fragmentation pattern of polyporenic acid-C (47)

The fragmentation pattern of compound 47 is shown in scheme E. These cleavages substantiate the fact that tetracyclic triterpenoids (like steroids) substituted at C-17 show ring D cleavage with loss of C-15, C-16 and C-17, together with their substituents. However, the formation of base peak has been rationalized by a sequence of reactions which do not involve ring D cleavage.

2.2.12 Identification of component L as dehydrotumulosic acid (48)

Component L is a white solid, mp >300°C, which shows UV absorptions at 236 (logϵ 3.98), 243 (logϵ 4.14) and 251 (logϵ 3.90)nm. The three banded UV spectrum of component L is srikingly similar to the UV spectrum of polyporenic acid-C (47) suggesting that component L contains a heteroannular diene.

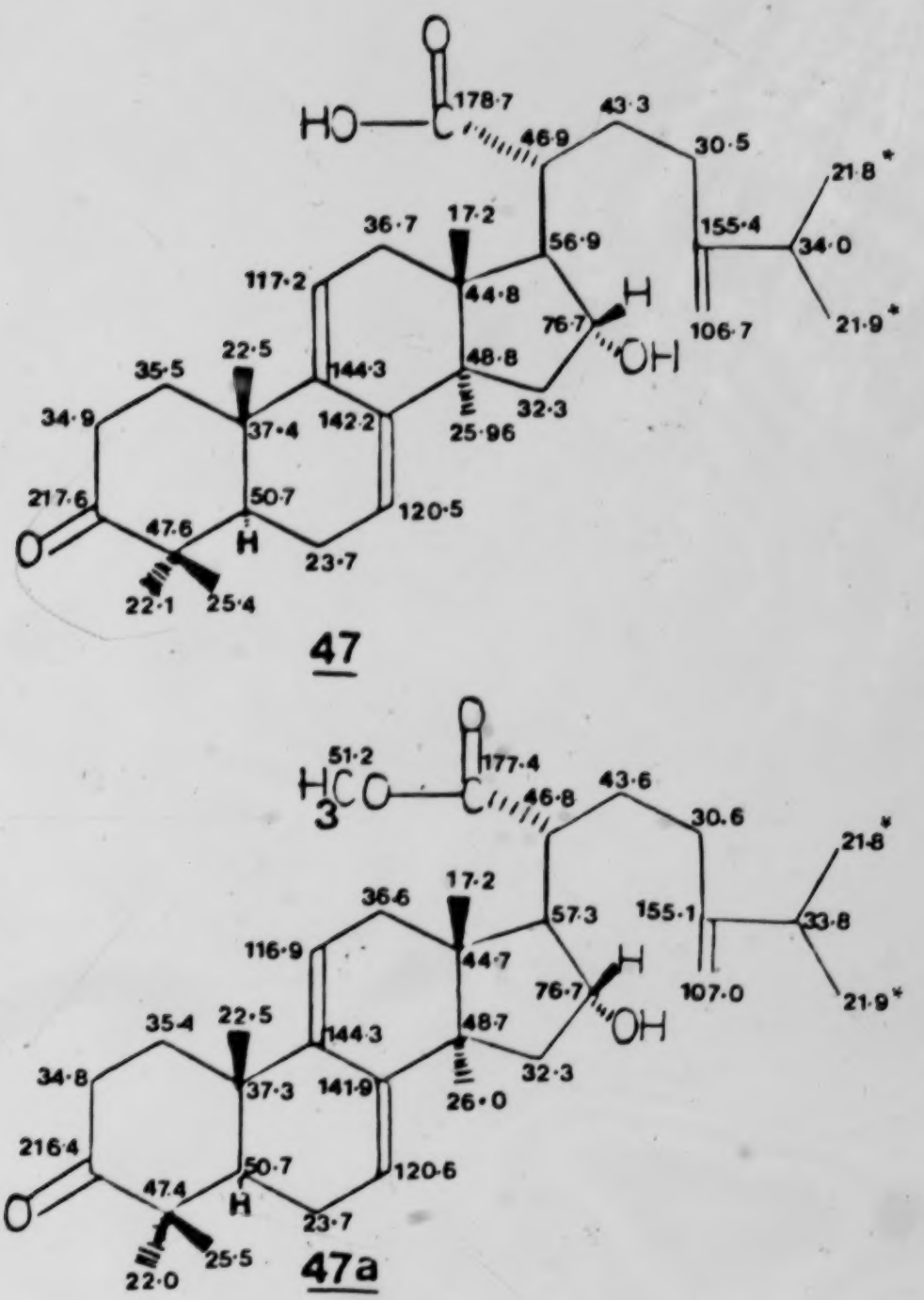
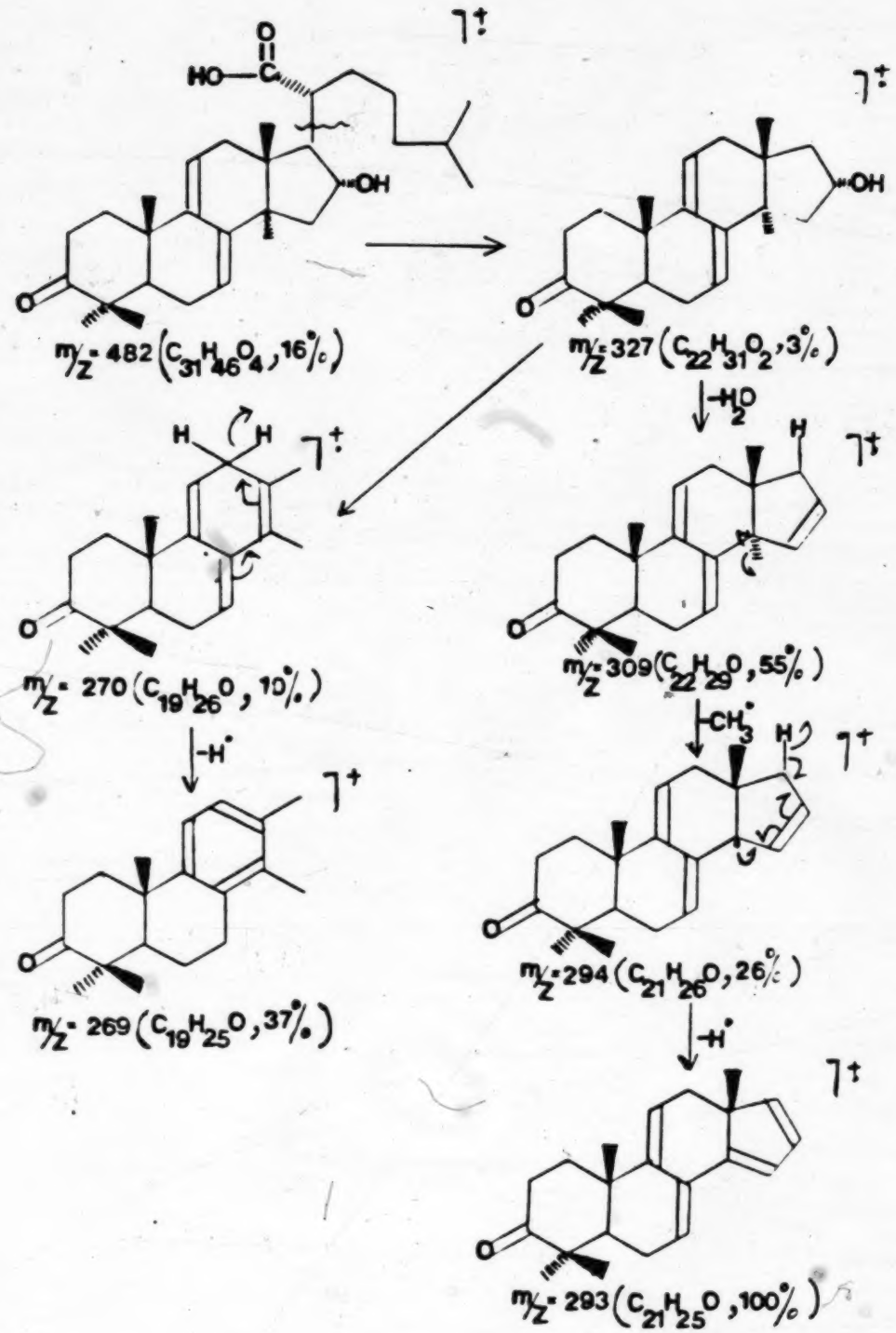
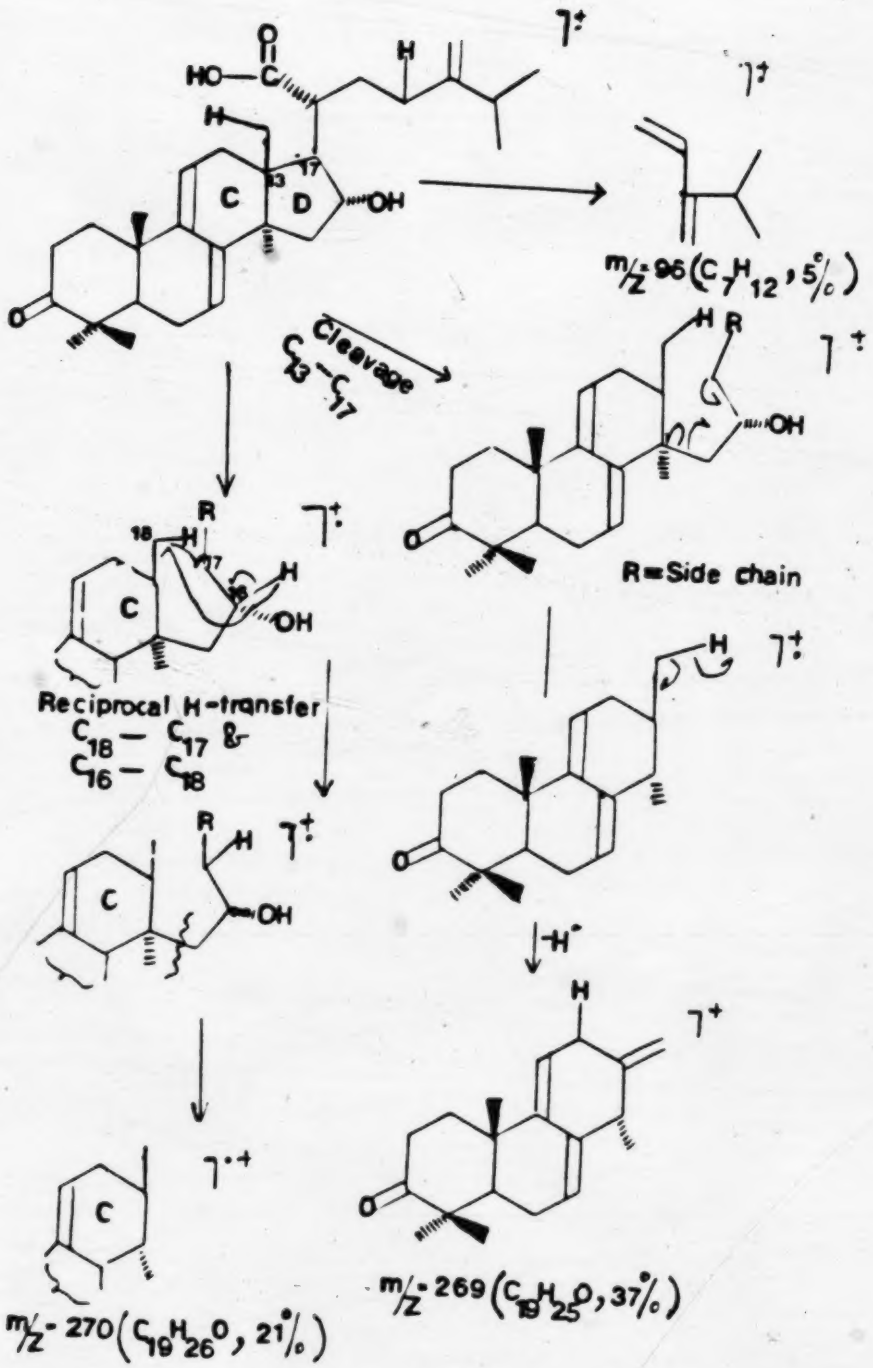


Figure 12 : 100 MHz ^{13}C NMR spectral data ($CDCl_3$) of polyporenic acid-C (47) and methyl polyporenate-C (47a) : can be interchanged.



Scheme E : The mass spectral fragmentation pattern of polyporenic acid-C (47).



Scheme E continued

The HREIMS of Component L indicates a parent peak of 484 amu corresponding to a molecular formula of $C_{31}H_{48}O_4$ and the presence of 8 unsaturation equivalents. The molecular formula of component L differs by 2 amu from polyporenic acid-C (47). The weak molecular ion was confirmed by CIMS using ammonia as a carrier gas.

The FTIR of component L shows the presence of a broad band extending from 3600-1900 cm^{-1}, a noticeable medium intensity peak at 3380 cm^{-1} and a strong peak at 1735 cm^{-1}, suggesting the presence of a carboxylic acid.

Component L (48) forms a monoester 48a (^1H NMR, δ 3.71, s, 3H, $COOCH_3$), the ^{13}C NMR of which shows the presence of 32 carbon atoms in the molecule; 8 quaternary carbons as singlets (s) at δ 176.1, 155.1, 145.7, 141.6, 48.6, 44.7, 38.7 and 37.5; 8 tertiary carbons as doublets at δ 121.0, 115.8, 78.9, 77.0, 57.4, 49.2, 46.7 and 33.8; 8 secondary carbons as triplets at δ 107.0, 43.6, 35.7, 35.4, 32.3, 30.6, 27.8, and 23.0 and 8 primary carbons as quartets at δ 51.2, 28.2, 26.2, 22.7, 21.9, 21.8, 17.2 and 15.8. Consideration of ^{13}C NMR signal multiplicities indicates the presence of 48 hydrogens directly attached to carbons in 48a. Since the ^{13}C NMR spectrum of component L was carried out on its methyl ester 48a, this suggests that component L must contain 45 hydrogen atoms directly attached to carbon atoms.

Component L forms a diacetyl derivative 48c [acetic anhydride-pyridine; ^1H NMR, δ2.06 (s, 3H), 2.05 (s, 3H)] and

this together with the formation of monoester 48a (CH_2N_2-$(C_2H_5)_2O$, ^1H NMR δ: 3.72, s, 3H) confirms the presence of only 3 exchangeable hydrogen atoms.

Since the molecular formula of component L differs by 2 amu's from polyporenic acid-C (47) it suggests that component L may be a dihydro derivative of polyporenic acid-C (47).

In support of this observation is the mass spectral fragmentation pattern of component L (Scheme F) which shows a base peak at m/z 295 ($C_{21}H_{27}O$). The base peak is 2 amu's greater than that observed in the fragmentation pattern of 47.

The FTIR of component L shows the vinylic methylene absorption at 1640, 884 cm^{-1} and this together with its UV data indicates that the three double bonds present in polyporenic acid-C (47) are also present in component L. Furthermore, the absorption maxima at 1714 (s) cm^{-1} characteristic of six-membered ring ketone in polyporenic acid-C (47) is absent in the FTIR of component L. This evidence suggests that component L and component K are related as hydroxyl and ketone congeners.

Further support for the relationship of component L and component K as hydroxyl and ketone congeners is borne out from the ^{13}C and ^1H NMR spectra of component L. In the ^{13}C NMR spectrum of component L no ketone carbonyl resonance is observed but a carbinol carbon at δ78.9 (d) characteristic of C-3, β-OH e.g., δ C-3 lanosterol 78.3 is observed.

The 'H NMR of component L is strikingly similar to that of polyporenic acid-C (47). The 'H NMR of component L exhibits a signal at δ3.2 (m, 1H), which sharpens (dd, J=4 and 11 Hz, 1H) on deuteration. This signal is observed for many tetracyclic triterpenoids (108) and is assigned to H-3 α. Figure 13 shows 'H NMR spectrum of compound 48 and Figure 14, 14a show the 'H NMR spectrum of 48a and its D_2O exchange spectrum respectively.

The 'H NMR methyl resonances of component L and its derivatives are depicted in Table 14.

The ^{13}C NMR spectrum of methyl dehydrotumulosate (48a) has been assigned and resonance values are listed in Figure 15.

2.2.13 The mass spectral fragmentation pattern of dehydrotumulosic acid (48)

The mass spectral fragmentation of dehydrotumulosic acid (48) is quite similar to polyporenic acid-C (47). The flow-chart of some of the fragmentation sequences of compound 48 are summarized in Scheme F.

2.2.14 Correlation of component K and component L

The relationship between component L and component K as hydroxyl and ketone congeners was confirmed by correlation experiments I and II. Thus $NaBH_4$ reduction of a methanolic solution of polyporenic acid-C (47) or methyl polyporenate-C (47a) afforded the corresponding alcohols, 48 and 48a

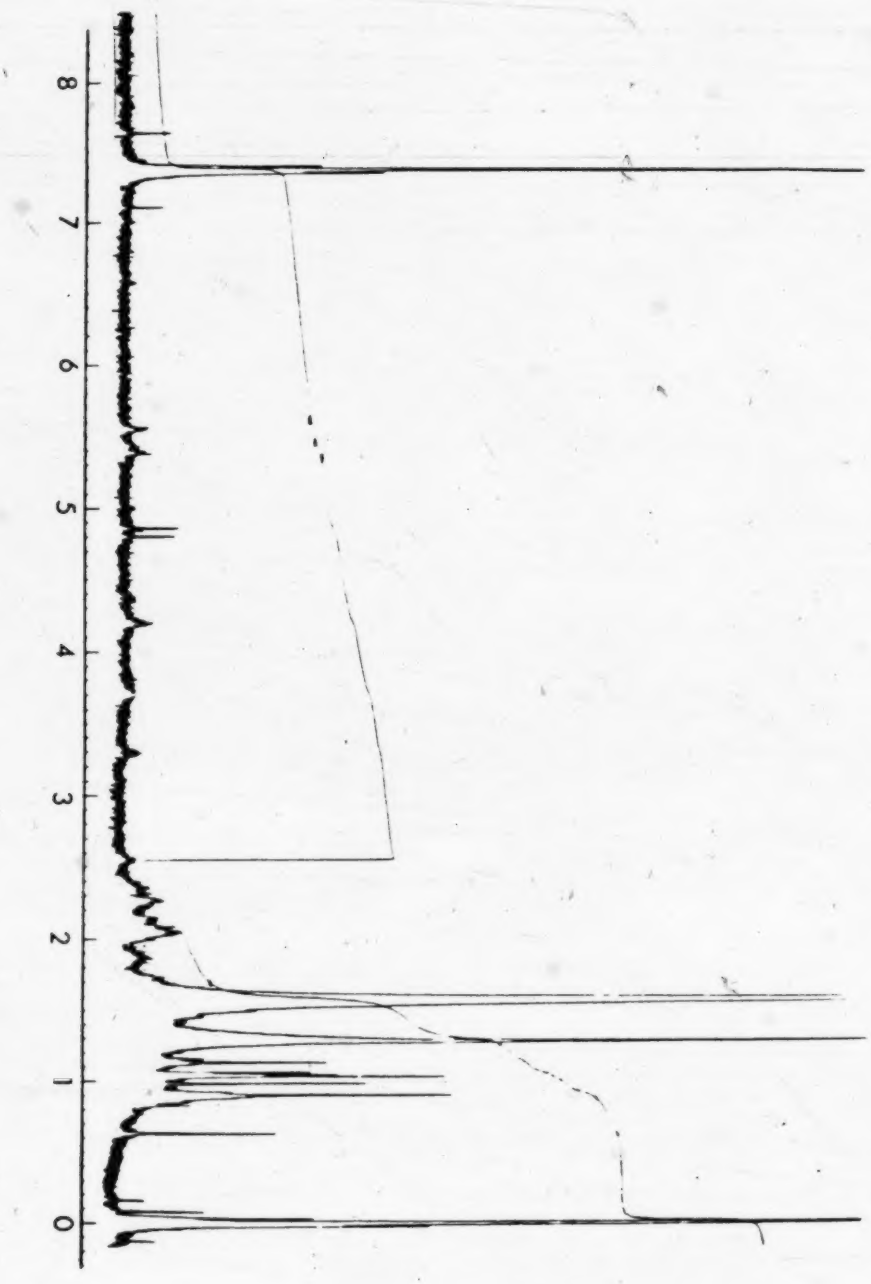


Figure 13 : 400 MHz ^{1}H NMR spectrum ($CDCl_3$) of dehydrotumulosic acid (48)

Figure 14 : 400 MHz ^1H NMR spectrum ($CDCl_3$) of methyl dehydrotumulosate (48a)

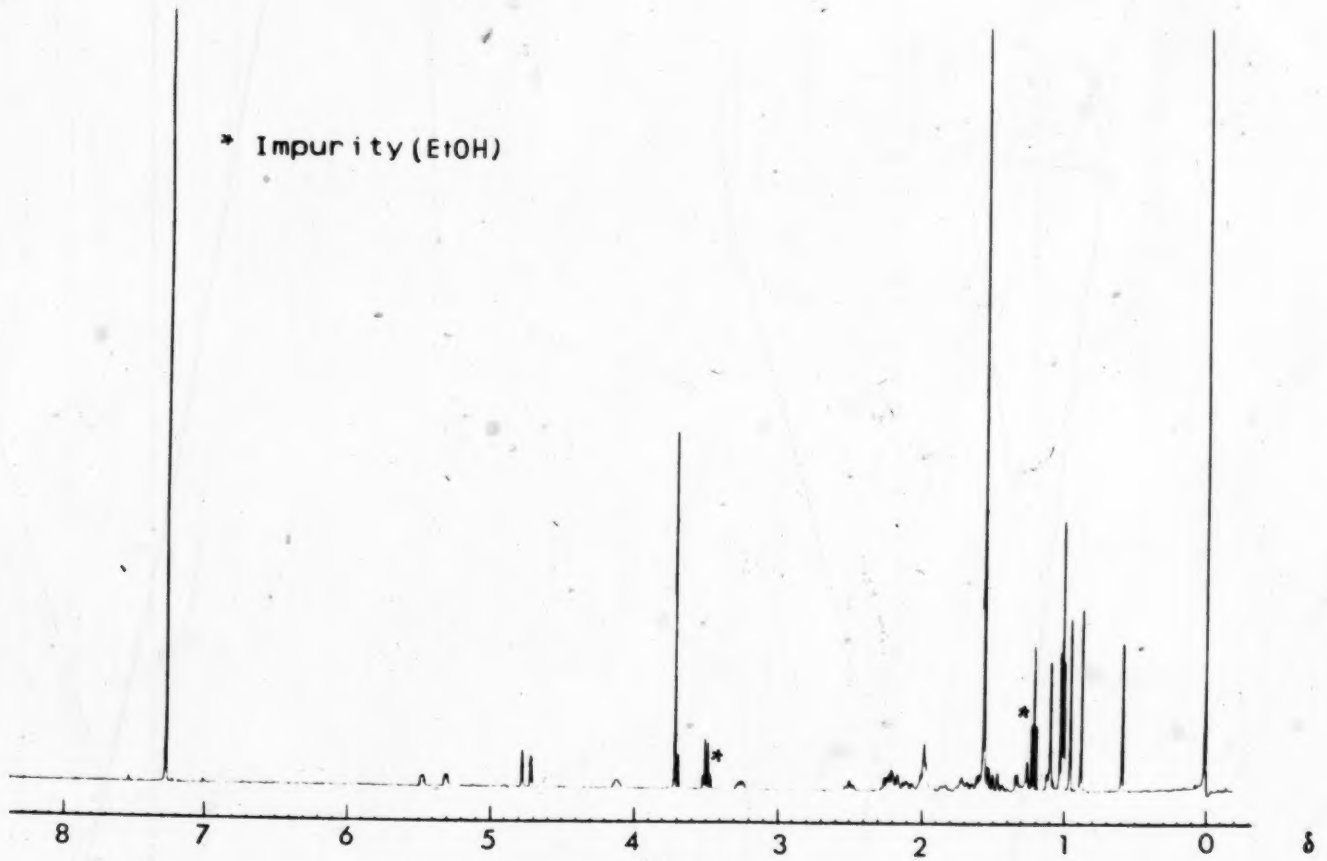


Figure 14a : 400 MHz D_2O exchange ^1H NMR spectrum ($CDCl_3$) of methyl dehydrotumulosate (48a)

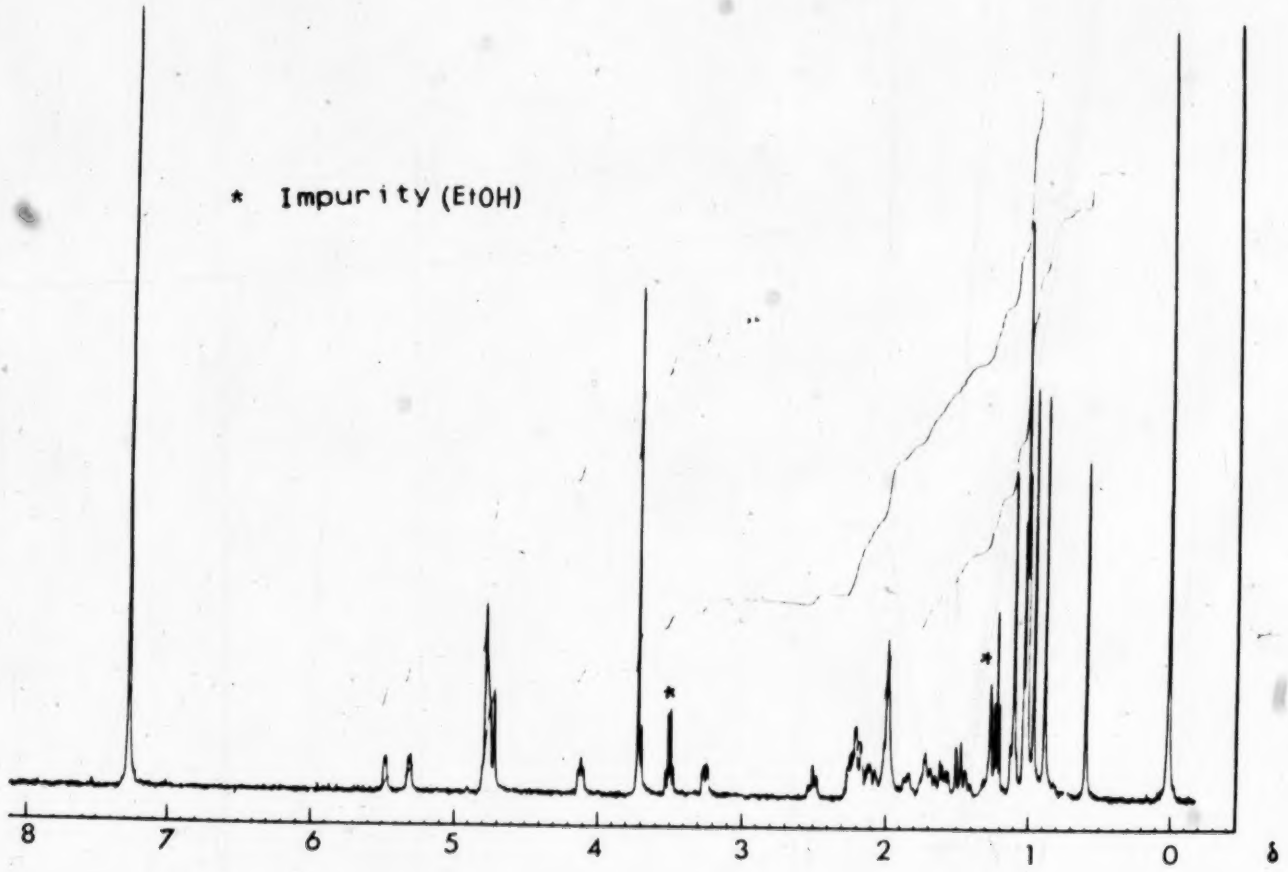


Table 14

^{1}H NMR methyl resonances of dehydrotumulosic acid (48) and its derivatives

Compound	C-18CH,	C-19CH,	C-26CH, and C-27CH, (J=7 Hz)		C-30CH, (4α)	C-31CH, (4β)	C-32CH,
48	0.61, s	1.03, s	1.00, d	1.02, d	0.89, s	0.97, s	1.12, s
48a	0.60, s	1.03, s	1.00, d	1.02, d	0.89, s	0.97, s	1.10, s
48b	0.63, s	1.01, s	1.00, d	1.02, d	0.88, s	0.96, s	1.04, s
48c	0.63, s	1.03, s	1.00, d	1.02, d	0.88, s	0.97, s	1.05, s

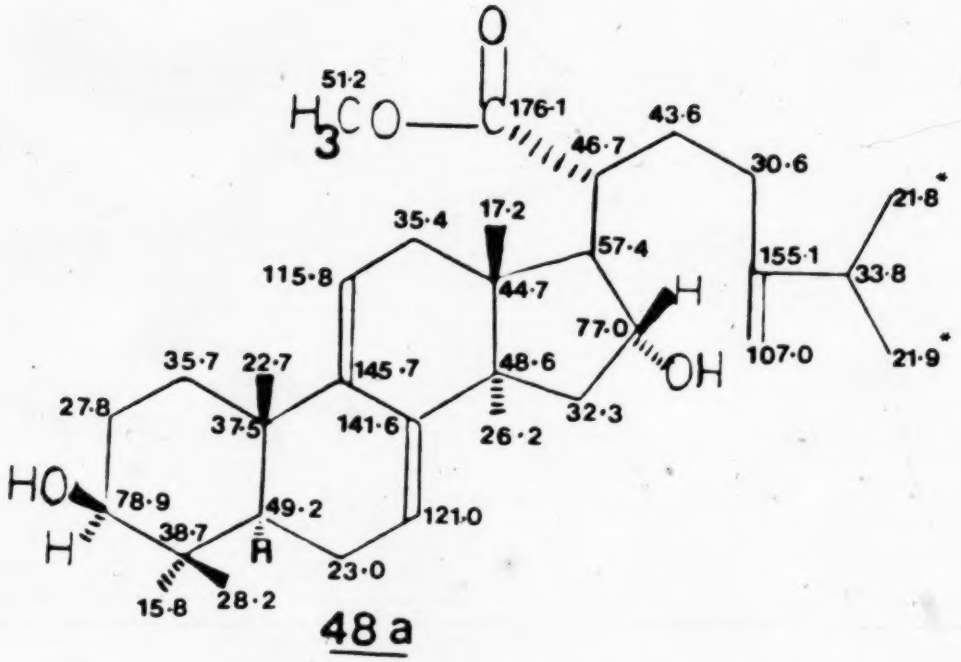
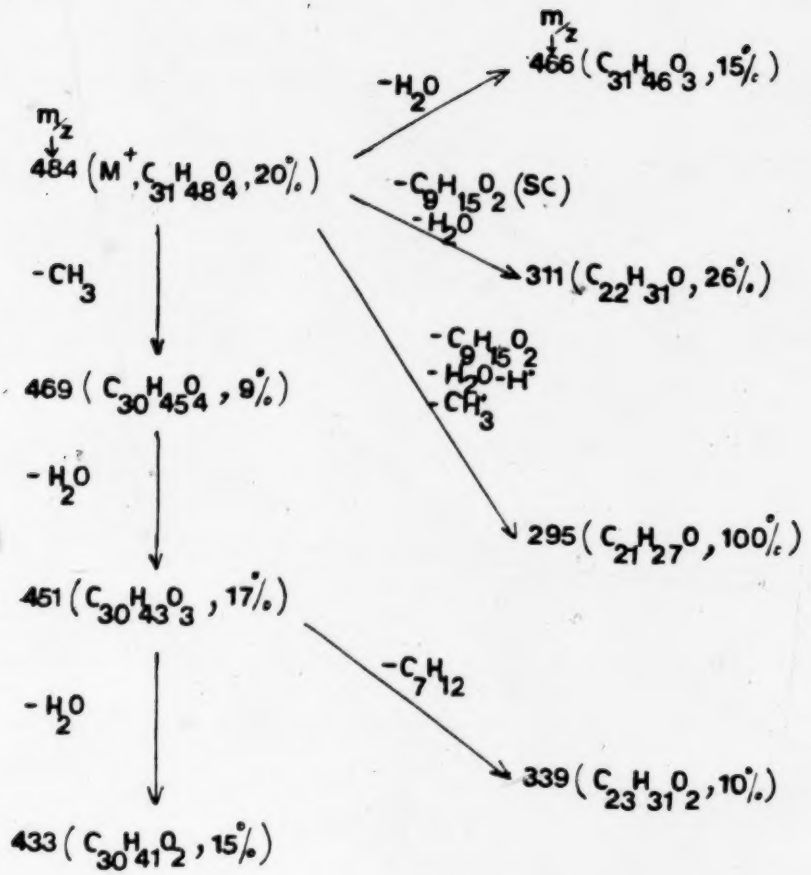


Figure 15 : 100 MHz ^{13}C NMR spectral data ($CDCl_3$) of methyl dehydrotumulosate (48a) : * can be interchanged



Scheme F : The mass spectral fragmentation pattern of dehydrotumulosic acid (48).

respectively which were identical in every respect ('H, MS, TLC, IR) with naturally occurring dehydrotumulosic acid (48) and methyl dehydrotumulosate (48a).

Dehydrotumulosic acid (48) has been reported earlier by Pinhey *et al.* (85). However, this report lacks complete information on this acid. Furthermore, dehydrotumulosic acid (48) has been claimed to be found in *Daedalea dickinsii, Melanporia juniperina, M. rosea, Trametes dickinsii, T. feei and T. lilacinogilva* (82).

Triterpenoids of the lanostane group containing the 7,9'' diene system are frequently accompanied by the corresponding (8-ene) dihydro compounds from which they have not been readily separated (109, 110). At one time lanosta-7,9'' dienes were considered to be artifacts resulting from the autooxidation of the 8-enes. Lanost-8-en-7-ones are well recognized autooxidation products of lanosta-7,9''dienes (110). Compound 49 having $\Delta^{7,9(11)}$ diene but without the steroidal side chain has been isolated by column chromatography (87). In addition, perenniporiol derivatives containing Δ^{8} or $\Delta^{7,9(11)}$ dienes and a six-membered 22, 26-epoxy side chain have recently been (87) separated by HPLC (Unisil QC 18). Our isolation of lanostane compounds having the $\Delta^{7,9(11)}$ diene system and a steroidal-type side chain (e.g., 47 or 48 by slow column chromatography has been demonstrated for the first time.

With the notable exception of agnosterol, dihydroagnosterol, polycarpol, and the sapogenins related to

the marine invertebrates (sea cucumbers) of the Phylum Echinodermata. e.g., *Bohadschia koellikeri* (Seychelles Archipelago in Indian Ocean), *Actinopyga agassizi* (Caribbean Coast line) and *Halodeima grisea* (Brazilian Coast line), the naturally occuring lanosta-7,9''dienes are metabolic products of fungi of the family Basidiomycetes.

2.2.15 The biological activity of the metabolites of *F. roseus*

Two different bioassays were used to screen for biological activity (antibacterial and antifungal).

Method A : (Modified Kirby-Bauer method)

This method was employed for testing compounds or mixture of compounds against bacteria or the fast growing fungus *Candida albicans* . In this method the petri dish (Muller-Hinton agar plates) was swabbed with an inoculum prepared by growing the test microorganism in sterile liquid media (Muller Hinton or PDB). The liquid inoculum was spread on the surface of the agar plate using a sterile cotton swab. The disk, impregnated with the compound/or mixture of compounds was applied to the petri dish after the inoculum is absorbed in the agar. The results of these tests are recorded after 24 hours. A test is positive when the growth of the microorganism is inhibited in a zone surrounding the impregnated disk.

The broth and mycelial extracts of cultures of *F. roseus* and the different extracts of the sporocarp were

tested against a variety of fast growing microorganisms. These test results reported after 24 hours as zone diameters of inhibition for 5% solutions of broth cultures, sporocarp extracts, and their pure components are listed in Table 15 and Table 16.

Method B : (Plug method)

This method was employed to screen *F. roseus* for antagonistic effect against other test fungi. In this method Potato Dextrose Agar (PDA) plates were prepared in a standard way. *F. roseus* and the test fungi were grown on separate agar plates. Plugs of similar size, were used to remove inoculums from *F. roseus* and the test fungi. These were carefully placed on opposite ends of a PDA plate. After suitable incubation, the antagonistic effect between *F. roseus* and the test fungus was observed by checking inhibition zone and/or looking for broken hyphae or thinner hyphal growth under microscope. The following test fungi were used:

1. *Verticladiella wageneri* C-728
2. *Verticladiella wageneri* UAMH # 4904
3. *Ceratocystis ulmi* UAMH # 5030
4. *Talaromyces flavus* ATCC # 5220
5. *Mycena citricolor* ATCC # 12570
6. *Ceratocystis minor* C-839
7. *Ceratocystis minor* C-248
8. *Ceratocystis clavigera* C-838
9. *Lachnellula* C-6983

Table 15

Antimicrobial bioassay of the culture extracts

List of cultures	V-8 broth	V-8 mycelia	Malt broth	Malt mycellia
Enterobacter cloacae	-	-	8	-
Escherichia coli	-	-	10	-
Klebsiella pneumoniae	-	-	-	-
Proteus vulgaris	-	9	8	-
Pseudomonas aeruginosa	-	-	-	-
Salmonella typhimurium	-	-	-	-
Serratia marcescens	-	-	-	-
Staphylococcus aureus	10	-	10	-
Staphylococcus epidermidis	-	-	-	-
Streptococcus pyogenes	-	10	10	-
Candida albicans	-	-	8	-

Note: Numbers above indicate the measurement of zone diameter (mm) using 6 mm diameter disks.

- No activity

Table 16

Antimicrobial bioassay of the sporocarp extracts, pure components and their analogues

List of cultures	Extract I	Extract IIW	Extract IIs	Extract IIb	47	47a	47b	48
Enterobacter cloacae	-	-	-	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-	-
Klebsiella pneumoniae	-	-	9	-	-	-	-	-
Proteus vulgaris	-	8	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-
Salmonella typhimurium	-	-	-	-	-	-	-	-
Serratia marcescens	-	-	-	-	-	-	-	-
Staphylococcus aureus	8	8	-	14	12	12	-	8
Staphylococcus epidermidis	-	-	8	10	8	-	-	-
Streptococcus pyogenes	8	-	-	-	-	-	-	-
Candida albicans	10	12	12	12	10	10	8	8

Note: Numbers above indicate the measurement of zone diameter (mm) using 6 mm diameter disks.

- No activity

10. *Armillari mellea* (Canadian strain)

None of the fungi listed above were inhibited by *F. roseus*. Only those fungi showing inhibition/antagonistic effect are suitable candidates for the fungal bioassay and hence further fungal bioassay experiments with various extracts of *F. roseus* were not carried out.

3. CONCLUSIONS

1. The work that has been completed represents only a beginning of studies that can be carried out with this fungus. We have concentrated on a study of the metabolites produced by the sporocarp of the fungus and by certain liquid cultures. We observed variation between V-8 and malt broth culture media and have also found that the sporocarp extracts are extremely complex. The malt broth extract produced simple hydroxyphenolic compounds and no triterpenoids whereas, the sporocarp extracts contained a variety of triterpenoids. Phenolic compounds are present in the sporocarp in large amounts but these were not studied in detail.
2. The sporocarp produced compounds related to those present in wood, suggesting that the compounds obtained may be fungal modifications of components of the tree.
3. The conflicting and incomplete data reported in the literature regarding polyporenic acid-C (47) and dehydrotumulosic acid (48) has been resolved. This allows assignment of their structures with certainty.
4. Although some biological activity was detected for some of the metabolites, further investigation of this aspect will be necessary.

4. EXPERIMENTAL

The distilled water used during the fungal culturing process was redistilled using an all glass apparatus. The fungus *Fomes roseus* (Strain B-58, UAMH # 4787), was grown in still culture at room temperature on an aqueous liquid medium containing either malt extract (Difco) 25.0 g, dextrose 13.0 g, and peptone 0.7 g/liter or 10% clarified V-8 juice and 1% glucose per liter. Malt extract is an infusion of malt, dried and powdered under conditions which conserve its diastatic activity and contains maltose 52.2%, dextrose 19.1%, sucrose 1.8%, dextrin 15.0%, other carbohydrates 3.8%, protein 4.6%, ash 1.5%, and moisture 2.0%. V-8 juice was filtered through Celite before use and is essentially a blend of eight vegetable juices (Campbell Soup Company Ltd., Toronto, Canada). All the solvents used for metabolite extraction and chromatographic separations were reagent grade and were distilled prior to use. Skellysolve B refers to Skelly Oil Company light petroleum, bp 62-70°. For centrifugal liquid chromatography (CLC) a Chromatotron (Harrison Research Product, Palo Alta, California) packed with Terochem thin layer chromatography silica gel G, was used as an adsorbent. For column chromatography (E. Merck, silica gel 60, 70-270 mesh, Brinkman Inst. Ltd.) was used while (E. Merck) silica gel 60 (230-400 mesh, ASTM) was used for flash chromatography. Terochem silica gel G containing 1% of Retma P-1 electronic phosphor (General Electric, Cleveland) 0.25-0.50 mm layer

thickness and E. Merck precoated thin layer chromatography plates 20 x 20 cm plate, 0.25-0.50 mm layer thickness, were utilized for preparative thin layer chromatography. Thin layer chromatographic analysis (TLC) was done on E. Merck (BDH) precoated aluminium sheets precoated with silica gel F_{254} (0.25 mm layer thickness). The chromatograms were examined under ultraviolet light (254, 354 nm) or developed with iodine vapors. For analytical TLC, the visualizatiton of the chromatograms were carried out by spraying with 10% sulphuric acid in water or with a solution of 5% phosphomolybdic acid containing 0.5% of ceric sulphate in 5% sulphuric acid in water, followed by careful charring on a hot plate (approx. 300°C). All spots char blue using the latter spray reagent with a yellowish-blue background. Melting points were determined on a Fischer-Johns melting point apparatus and are uncorrected. All thin layer Rf values refer to distance travelled on unactivated (E. Merck) precoated aluminium TLC plates (Silica gel 60, F_{254}) and the TLC chambers were unequilibrated. High resolution electron impact mass spectra (HREIMS) were recorded on an AE I MS-50 mass spectrometer. Chemical ionization mass spectra (CIMS) and low resolution electron impact mass spectra (LREIMS) were obtained using an AE I, MS-4 mass spectrometer. CIMS was obtained with ammonia as a carrier gas. Fast atom bombardment (FAB) spectra were obtained using a MS-50 spectrometer. The data were processed in DS-50 and DS-9 computers. Fragment ions reported by m/z (molecular formula,

relative intensity, fragmentation characteristics wherever applicable, have been determined by HREIMS. Unless diagnostically significant only peaks at least 20% as intense as the base peak are reported. Molecular formulae were determined by HREIMS from the corresponding molecular ion and were calculated using C=12.000 H=1.0078 and O=15.9949. The base peak is referred to as (B) and side chain as (SC) wherever deemed necessary. Fourier transform infrared (FTIR) spectra were recorded on a Nicolet 7199 FT interferometer. Single scan infrared (IR) spectra were recorded on a Perkin - Elmer 297 infrared spectrometer. Abbreviations used for reporting infrared (IR) data are the following : (s) strong, (b) broad, (m) medium, (sh) shoulder, (w) weak, and (vw) very weak. Ultraviolet (UV) spectra were obtained on a Unicam SP-1700 Ultraviolet Spectrophotometer and Hewlett Packard 8450 A Diode Array Spectrophotometer attached with Hewlett Packard HP-85 Computer. Either ϵ or $\log\epsilon$ values are reported for UV. Optical rotations were recorded on a Perkin-Elmer 141 Polarimeter while optical rotatory dispersion (ORD) and circular dichroism (CD) spectra were recorded on a Durrum Jasco ORD/UV - 5 (SS-20 modification) recording spectro polarimeter.

High field ^1H NMR and ^{13}C NMR spectra were recorded on Bruker WH-200 and/or WH-400 spectrometers with an Aspect 2000 computer system. Tetramethylsilane (TMS) was used as an internal standard. Abbreviations for reporting NMR data are

the following : (s) singlet, (d) doublet, (t) triplet, (vt) virtual triplet, (q) quartet, (m) multiplet, (cm's) complex multiplets, and (br) broad.

4.1 Malt culture broth

4.1.1 Cultivation of *Fomes roseus*

The strain of *Fomes roseus* was maintained on 2% malt agar slant culture at 4°C. Shake cultures of the fungi were obtained by transferring small pieces of mycelium from the slant culture to 300 mL Erlenmeyer flasks containing 150 mL of sterile 5% malt broth. After two weeks at 17°C, the shake cultures were ready to be used as an inoculum. Aliquots of inoculum (25mL) were transferred to Fernbach flasks, each containing sterile malt broth extract (1L). The flasks were maintained undisturbed at room temperature for two growth periods; 4 weeks and 7 weeks. After several days, whitish mycelia appeared on the surface of the still culture. By the beginning of the 3rd week brownish pink to rose coloured mycelium appeared. The quantity of the extracts obtained from the growth studies have been described in Table 4.

4.1.2 Extraction of the metabolites from 4 and 7 weeks malt culture broth

The still culture growth was harvested after 4 and 7 weeks. The broth was separated from mycelium by filtration through cheese cloth. The broth was concentrated at reduced

pressure (5 L and 9 L to 500 mL), then extracted with ethyl acetate in a continuous liquid-liquid extractor for 24 hours. The broth extract yielded 320-1575 mg/L of crude metabolites. The mycelium (from 5 L and 9 L), was air dried for three days, then extracted with ethyl acetate in a Soxhlet extractor. The ethyl acetate extracts were dried (anhydrous Na_2SO_4) filtered, and concentrated *in vacuo* at 35-37°C to give 170-810 mg/L of mycelial extract.

4.1.3 Separation of the metabolites from 4 weeks old malt culture broth

4.1.4 Isolation and identification of linoleic acid (41, component A)

The broth extract (0.64 g) was subjected to flash chromatography over silica gel (column diameter = 25 mm, SiO_2 depth = 20.5 cm) using Skellysolve B for packing the column. A gradient solvent system (Skellysolve B: ethyl acetate 2:1 to Skellysolve B: ethyl acetate, 1:1) was employed. Fourteen fractions as mixtures were collected. One of the fractions (15 mg) containing pinkish colored material was further fractionated by CLC (Chromatotron) using polarity gradient elution by adding Skellysolve B in ratios 2:1, 1:1, and 1:2 to diethyl ether (100 mL each). One mL fractions were collected. One such fraction contained component **A** as a waxy yellowish-white semisolid (1.5 mg). Component **A** was identified as linoleic acid 41 on the basis

of its spectral properties and by comparison with an authentic sample.

TLC: Rf 0.38 (Skellysolve B : diethyl ether, 1:1) and 0.59 (Chloroform: methanol, 100:1).

HREIMS (Probe, 100°C) m/z (mol. for., rel.int): $C_{18}H_{32}O_2$ [M^+, calcd : 280.2394; found 280.2399] (12), 220($C_{16}H_{28}$, 5, $M^+-C_2H_4O_2$), 108(C_8H_{12}, 36), 94(C_7H_{10}, 20), 60($C_2H_4O_2$, 25, $M^+-C_{16}H_{28}$), and 55 (C_4H_7, 100, B).

FTIR ($CHCl_3$ cast) : 3600-2400(b), 2930(s), 2860(s), 1710(s), 1462(w), 1405(w), 1280(b,w), 940(m,b), and 740(m,b) cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$) δ: 11.87 (br.s 1H, COO<u>H</u>) 5.37 (m, 4H, (-C<u>H</u>=C<u>H</u>)$_2$), 2.80 (m, 2H, -HC=CH-C<u>H</u>$_2$-CH=CH) 2.36 (t, J=6 Hz, 2H, -C<u>H</u>$_2$-COOH), 2.04 (m, 4H, -C<u>H</u>$_2$-HC=CH), 1.6 (m, 2H, -C<u>H</u>$_2$-CH$_2$ -COOH), 1.37 (br.m 14H, -C<u>H</u>$_2$-) and 0.94 (vt, 3H, -C<u>H</u>$_3$).

4.1.5 Separation of metabolites from 7 weeks old malt culture broth

4.1.6 Isolation of component B and component C

The broth extract (1.75 g) was suspended in distilled water (100 mL) and extracted with Skellysolve B (2 x 100 mL) to remove most of the fatty acids and triglycerides (800 mg). The Skellysolve B extract was not analysed. The aqueous extract was extracted with diethyl ether (2 x 500 mL, mechanical stirring). The diethyl ether extracts were combined and concentrated *in vacuo* to give a brownish-white

residue. The bulk of the residue was redissolved in $CHCl_3$. The remaining $CHCl_3$ insoluble mixture of compounds (50 mg) was subjected to flash chromatography over silica gel (column diameter = 30mm, SiO_2 depth = 16 cm) using benzene : diethyl ether (2:1, 1:1, 0:1, 300 mL each), then methanol : diethyl ether (1:1, 150 mL). The earlier fractions of the column gave component **B** (6 mg) while latter fractions gave an impure fraction (10 mg). Further purification was achieved by flash chromatography over silica gel (column diameter = 10 mm, SiO_2 depth = 15 cm) using 10% of CH_3OH in $CHCl_3$ as eluting solvent system to give component **C** (4 mg) as a white solid.

4.1.7 Identification of component B as p-hydroxyphenyl acetic acid (42)

Component **B** is a colorless compound soluble in methanol, benzene, acetone and diethyl ether, mp 133-138°C.
TLC: Rf 0.31 (benzene: diethyl ether, 1:1)
UV λmax. (CH_3OH), 275 (ϵ 8025) nm.
HREIMS (Probe, 100°C) m/z (mol.for., rel.int): $C_8H_8O_3$ [$M^{\cdot}$, Calcd : 152.0471; found: 152.0473] (29), 107 (C_7H_7O, 100, B, $M^{\cdot}-CO_2H$), 79 (C_6H_7, 2, B-CO), and 77 (C_6H_5, 15, B-CO-2H).
FTIR (CH_3OH-$CHCl_3$ cast): 3540-2400 peak at 3258(s), 1707(s), 1610(m), 1598(m), 1518(m), 1440(m), 1405(m), 1362(w), 830(m), 820(m), 780(m), 650(m) and 540 (m) cm^{-1}.
^1H NMR (400.1 MHz, Acetone-d_6) δ: 12.68 (d, J=1.5 Hz, 1H), 12.3-10.6 (br.s, 1H - COO**H**), 7.14(d, J=8 Hz, 2H), 6.79 (d,

J=8 Hz, 2H) and 3.53(s, 2H, C_6H_5-C<u>H</u>$_2$-).

4.1.8 Esterification of component B

Compound <u>42</u> (5 mg) was dissolved in ethereal diazomethane (3 mL). The reaction mixture was allowed to stir for 3 hours at room temperature. The reaction was monitored by TLC. Evaporation of the solvent *in vacuo* gave oily compound <u>42a</u> in quantitative yield.

TLC : Rf 0.58 (benzene : diethyl ether, 1:1)

HREIMS (Probe, 150°C) m/z (mol.for., rel.int.): $C_9H_{10}O_3$ [M^+, calcd: 166.0627; found: 166.0630] (25), 107 (C_7H_7O 100, M^+-CO_2CH_3), 79 (C_6H_7, 2, B^+-CO) and 77 (C_6H_5, 13, B^+-CO-2H).

FTIR ($CHCl_3$ cast): 3450(b), 1735(s), 1618(m), 1599(m), 1515(s), 1440(m), 1405(w), 1264(s), 1240(s), 1223(s), 1163(s), 1100(m), 1010(m), 826(m), 800(m), and 790(w) cm^{-1}.

^{1}H NMR (200MHz, Acetone-d_6) δ: 7.13 (d, J=8 Hz, 2H), 6.79 (d, J=8 Hz, 2H), 3.66 (s, 3H, -COOC<u>H</u>$_3$) and 3.54 (s, 2H, -C<u>H</u>$_2$-$COOCH_3$)

4.1.9 Acetylation of compound <u>42a</u>

Compound <u>42a</u> (4.5 mg) was stirred with pyridine (1.5 mL) and acetic anhydride (2.5 mL) at room temperature for 4 hrs. The reaction mixture was diluted with ice cold water (25 mL), then extracted with ethyl acetate (20 mL). The ethyl acetate extract was washed successively with 5% HCl (2 x 10 mL), 5% NaOH (2 x 10 mL), and brine (2 x 5 mL). The ethyl acetate extract was then dried over anhydrous Na_2SO_4,

filtered and concentrated under reduced pressure to give compound 42b as an oil in quantitative yield.

TLC: Rf 0.73 (benzene : diethyl ether, 1:1)

HREIMS (Probe, 150°C) m/z (mol. for., rel. int.): $C_{11}H_{12}O_4$ [$M^{\cdot}$, Calcd : 208.0732; found: 208.0736] (12), 165 ($C_9H_9O_3$, 80, $M^{\cdot}-COCH_3$), 149 ($C_9H_9O_2$, $M^{\cdot}-COOCH_3$), 107 (C_7H_7O, 100(B), $M^{\cdot}-C_4H_5O_3$), 79 (C_6H_7, 5, $B^{\cdot}-CO$), and 77 (C_6H_5, 14, $B^{\cdot}-CO-2H$).

FTIR ($CHCl_3$ cast): 2950(m), 2852(m), 1758(s), 1742(s), 1610(w), 1594(w), 1563(w), 1509(w), 1436(m), 1370(s), 1313(w), 1219(s), 1162(s), 1101(w), 1075(w), 1039(s), 932(m), 912(s), 849(m), 818(m), 656(m), 597(m) Cm^{-1}.

^1H NMR (200 MHz, Acetone-d_6 δ: 7.19 (d, J=8 Hz, 2H), 6.90 (d, J=8 Hz 2H), 3.69 (br.s, 3H, $COOC\underline{H}_3$), 3.59(br.s, 2H), and 2.11 (s, 3H, $-OCOC\underline{H}_3$).

4.1.10 Identification of component C as p-hydroxybenzoic acid (43)

Component C is a white solid crystallizing as white prismatic crystals ($CHCl_3$: CH_3OH), mp 208-211°C, readily soluble in ether and acetone.

TLC: Rf 0.28 (benzene: diethyl ether, 1:1) and 0.47 (benzene: acetone: acetic acid: 5:1:few drops).

UV λmax. (CH_3OH): 260 (ε 7600) nm.

HREIMS (Probe, 150° m/z (mol. for., rel. int.): $C_7H_6O_3$ [$M^{\cdot}$, Calcd : 138.0315; found 138.0317] (71), 121 ($C_7H_5O_2$, 100, $M^{\cdot}-OH$), 93 (C_6H_5O, 26, $M^{\cdot}-CO_2H$), and 65 (C_5H_5, 21,

$M^{+}-CO_2H-CO$).

FTIR ($CH_3OH-CHCl_3$ cast): 3600-1900 (b) peak at 3388 (s), 1674(s), 1608(s), 1595(s), 1516(m), 1446(m), 1424(m), 1386(w), 1364(w), 1316(m), 1290(m), 1246(m), 1169(m), 1130(w), 1100(m), 1010(w), 930(m), 854(s), 769(m), 695(w), and 619(m)cm^{-1}.

^1H NMR(400.1MHz, $CDCl_3$) δ: 7.91(d, J=8 Hz, 2H) and 6.91(d, J=8 Hz, 2H).

4.2 Sporocarp

4.2.1 Extraction of the metabolites from the sporocarp of *Fomes roseus*

The basidiocarps of *Fomes roseus* (Alb. and Schw. ex Fries)Cooke were collected and identified in August 1982 and August 1983 by Dr. R.S Currah. They were found growing on fallen *Picea* (Spruce) near Drayton Vally, Alberta. The basidiocarps were air dried and wood shavings adhering to the basidiocarp were removed with a knife. The sporocarps were then cut with a knife and finally ground to a powder in a Waring blender. The powdered fungus (75g) was transferred carefully into a thimble and extracted with Skellysolve B in a Soxhlet extractor (24 hrs., 1.5 L). The extract was collected and the residue further extracted with Skellysolve B (12 hrs., 1.5L) to remove Skellysolve B soluble compounds completely. The thimble contents were drained of Skellysolve B and the extracts were combined, filtered through Celite

and concentrated to yield Skellysolve B extract (4.2 g). Further basidiocarp extraction, was repeated with diethyl ether, ethyl acetate, methanol and water using the procedure described for Skellysolve B to yield 34 g, 2 g, 3 g, and 2.5 g of concentrated extracts, respectively. The spongy residue left over was discarded.

Solvent extraction of the basidiocarps at room temperature with mechanical stirring was also carried out. Powdered fungus (75 g) was allowed to stir with Skellysolve B (1.5L, 24 hrs.), then the Skellysolve B was decanted and concentrated. This procedure was repeated with diethyl ether, ethyl acetate, methanol, and water to yield the corresponding extract (Scheme B for yields of extracts). The solvent partitioning at room temperature under mechanical stirring gave comparable yields of crude extracts (see Table 6). Since the TLC profiles of the extracts from Soxhlet extraction and solvent extraction by mechanical stirring were identical, the corresponding extracts were combined for further separation work. The quantity of the crude metabolites has been described in Table 6.

4.3 Purification of metabolites

4.3.1 Isolation of Component D

Extract I (300mg) was applied on the top of a silica gel column (column diameter= 30 mm, SiO_2 depth = 20 cm) using Skellysolve B: benzene (2:1) as the column packing

solvent. Slow chromatography with a gradient solvent system Skellysolve B: benzene (2:1, 500 mL), Skellysolve B: benzene (1:1, 500 mL), and benzene: acetone (1:1, 500 mL) was employed. Sixteen fractions (150 mL) were collected. The ninth fraction was rechromatographed over silica gel (column diameter= 1.5 cm, SiO_2 depth = 15 cm) by flash chromatography using Skellysolve B: diethyl ether (2:1, 1:1, 1:2; 300 mL of each) as the gradient solvent system. Further purification of one middle fraction by flash chromatography (Skellysolve B: ethyl acetate) over silica gel gave (5mg) of pale yellowish oil which appeared to be a single component in a TLC analysis.

4.3.2 Identification of component D as triglyceride (44)

TLC: Rf (0.32, Skellysolve B: ethyl acetate, 85:15 and 0.5 in $CHCl_3$).

HREIMS (Probe, 275°C) m/z (mol.for., rel.int.): [M$^+$, none], 603 ($C_{39}H_{71}O_4$,31) 602 ($C_{39}H_{70}O_4$, 17), 601 ($C_{39}H_{69}O_4$, 10), 493 ($C_{30}H_{53}O_5$, 22), 339 ($C_{21}H_{39}O_3$, 25)

CIMS (m/z, rel. int.): 790 (3).

FTIR ($CHCl_3$ cast): 3010(w), 2953-2854(s), 1745(s), 1650(w), 1600(w), 1465(m), 1375(m), 1260(w), 1163(w), 1060(w) and 725(m) cm^{-1}.

^1H NMR (200MHz,$CDCl_3$) δ: 5.38 (m, 9H, coupled to δ4.24, 2.80 and 2.04, H-2 + 8 vinylic protons), 4.24 (2 x dd, ABX system, 4H J=5.0, 6.5 and 12 Hz, coupled to δ 5.38, 2.81 br.t J=6 Hz, 4H, doubly allylic protons, coupled to δ 5.38),

2.34 (br.t J=8 Hz, 6H, $-CH_2(C\underline{H}_2)CO_2R$), 2.06 (br.t, J=6 Hz, 10H, coupled to δ5.38), 1.66 (m, 8H, $C\underline{H}_2CH_2CO_2R$), 1.35(br.s, 46H, $-(CH_2)_n-$, and 0.94(vt, 9H, $-C\underline{H}_3$). No D_2O exchangeable proton observed.

4.3.3 Isolation and identification of linoleic acid (41) as component E

Slow chromatography of Extract Ia (100 mg) over silica gel (column diameter= 20 mm, SiO_2 depth = 16 cm) was performed using ($CHCl_3$: CH_3OH, 99:1) as eluant. A major fraction (25 mg) was repeatedly chromatographed by flash chromatography (column dia = 20 mm, SiO_2 depth = 8 cm, eluant = Skellysolve B: $CHCl_3$, 1:8) to give component E, Rf 0.6 ($CHCl_3$: CH_3OH, 99:1) an oil. Component E was shown to have spectral properties identical with component A and was thus identified as linoleic acid (41).

4.3.4 Isolation of components F and G

Extract IB (200 mg) was chromatographed over silica gel (column diameter= 25 mm, SiO_2 depth = 15 cm) using gradient elution [Skellysolve B: $CHCl_3$, 9:1, 7:1, 5:1, and 3:1 (300 mL each)]. Three partially pure components were obtained. The most abundant component (30 mg) was subjected to flash chromatography over silica gel using Skellysolve B: $CHCl_3$ (4:1). Final purification was achieved by PTLC over silica gel (benzene: diethyl ether, 1:2, multiple elution) to afford component F as a white colorless solid (5 mg, mp

160-164°C).

The polar fraction (20 mg) was purified by flash chromatography over silica gel (column diameter = 10 mm, SiO_2 = 10 cm, eluant= benzene: diethyl ether, 1:1) Compound G was isolated as white crystals (3 mg, mp 169-174°C).

The least polar fraction (20 mg) was purified by flash chromatography over silica gel (column diameter= 25 cm, SiO_2=10 cm, eluant= Skellysolve B: $CHCl_3$, 5:1). Compound H (10 mg) was isolated as a pale yellow oil.

4.3.5 Identification of component F as ergosterol (45)

TLC: Rf 0.38 (benzene: diethyl ether, 1:1).

HREIMS (Probe, 200°C) m/z (mol.for., rel. int.): $C_{28}H_{44}O$ [M^+, Calcd: 396.3381; found: 396.3379] (100), 381 ($C_{27}H_{41}O$, 4, $M^+ - CH_3$), 378 ($C_{28}H_{42}$, 8, M^+-H_2O), 376 ($C_{28}H_{40}$, 5, $M^+-H_2O-H_2$), 364 ($C_{27}H_{40}$, 23, M^+-CH_3-OH) 363($C_{27}H_{39}$, 74, $M^+-CH_3-H_2O$), 337 ($C_{25}H_{37}$, 31, M^+-59), 271 ($C_{19}H_{27}O$, 15, M^+-SC, 253 ($C_{18}H_{25}$, 27, M^+-SC-H_2O), 211 ($C_{16}H_{19}$, 18) 157 ($C_{12}H_{13}$, 21), and 143 ($C_{11}H_{11}$, 26).

FTIR ($CHCl_3$ cast): 3400(b), 3040(w), 2951(s), 2933(s), 2869(s), 1730(b,w), 1660(w), 1600(w), 1467(m), 1378(m), 1068(m), 1038(m), 970(m), 837(m) and 801 (w) cm^{-1}.

UV λmax. (C_2H_5OH) : 262(ϵ 7200), 271(ϵ 9260), 282(ϵ 11800), and 292 (ϵ 6500) nm.

^1H NMR (200 MHz, $CDCl_3$) δ: 5.58 (dd, J=2.5 and 6 Hz, 1H), 5.39 (ddd, J=2.5, 2.5 and 6 Hz,1H) 5.23 (m, 2H), 3.68 (m, H-3 α, 1H), 2.6- 1.2 (21H, cm's methylene-methine protons),

1.08 (d, J=6.5 Hz, 3H), 1.0 (br.s, 3H), 0.95 (d, J=6.5 Hz, 3H), 0.89 (d, J=7 Hz, 3H) 0.87 (d, J=7 Hz, 3H), and 0.66 (br.s, C-18 $C\underline{H}_3$, 3H).

4.3.6 Identification of component G as ergosterol peroxide (46)

TLC: Rf 0.25 (benzene: diethyl ether, 1:1) and 0.3 (Skellysolve B: ethyl acetate, 85:15).

HREIMS (Probe, 250°C) m/z (mol. for., rel. int.): $C_{28}H_{44}O_3$ [M^+, Calcd: 428.3279; found: 428.3286] (5), 410 ($C_{28}H_{42}O_2$, 5, $M^+ - H_2O$), 396 ($C_{28}H_{44}O$, 100, M^+-O_2), 381 ($C_{27}H_{41}O$, $M^+-O_2-CH_3$), 378 ($C_{28}H_{42}$, 3, $M^+-O_2-H_2O$), 364 ($C_{27}H_{40}$ 23, $M^+-O_2-CH_3-OH$), 363 ($C_{27}H_{39}$, 31, $M^+-O_2-CH_3-OH_2$), 337 ($C_{25}H_{37}$, 13, M^+-O_2-59), 271 ($C_{19}H_{27}O$, 7, M^+-O_2-SC), 253($C_{19}H_{25}$, 12, M^+-SC-H_2O), 69 (C_5H_9, 39), and 55 (C_4H_7, 33).

FTIR ($CHCl_3$ cast): 3400(b), 2960(s), 2933(sh), 2860(s), 2380(vw), 2365(vw), 1730(w), 1600(b,w), 1470(m), 1390(m), 1080(m), 1050(m), 1038(m), and 980(m) cm^{-1}.

^1H NMR (400.1 MHz, $CDCl_3$) δ: 6.50 (d, J=8 Hz, 1H), 6.24 (d, J=8 Hz, 1H), 5.22 (dd, J=7 and 16 Hz, 1H), 5.14 (dd, J=8.2 and 16 Hz, 1H), 3.97 (m, $W_{1/2}$=14 Hz, 1H), 0.99 (d, J=6.2 Hz, 3H), 0.91 (d, J=6.5 Hz, 3H), 0.89 (s, 3H), 0.84 (d, J=7 Hz, 3H), 0.86 (d, J=7 Hz, 3H), and 0.83 (s, 3H).

4.3.7 Identification of component H as squalene (1)

HREIMS (Probe, 130°C) m/z (mol. for., rel. int.): $C_{30}H_{50}$[M^+, Calcd : 410.39; found: 410.3898] (9), 341 ($C_{25}H_{41}$, 5, M^+-69), 273 ($C_{20}H_{33}$, 3, M^+-137), 205($C_{15}H_{25}$, 3, M^+-205), 81 (C_6H_9, 47), and 69 (C_5H_9, 100).

FTIR ($CHCl_3$ cast): 3050(w), 2965(s), 2917(s), 2854(sh), 1667(w), 1446(m), 1381(m), 1150(w), 1107(w), 983(w), 834(m), and 740(w) cm^{-1}.

^1H NMR (400.1 MHz, $CDCl_3$) δ: 5.14 (m, 6H, olefinic), 2.04 (m, 20H, $-C\underline{H}_2-$) 1.66(br.s, 6H, $-C\underline{H}_3$) and 1.60(br.s, 18H, $-C\underline{H}_3$).

4.4 Purification of metabolites from extract II

4.4.1 Isolation of component J

Extract II contains a mixture of as many as twenty five compounds (TLC behaviour). Slow column chromatography (SiO_2, gradient 1-10% CH_3OH in $CHCl_3$, 500-1000 mL) of Extract II was unsuccessful because it gave complex mixtures as shown by TLC. Extract II was simplified by separation into a neutral fraction (extract IIB) and an acidic fraction (extract IIW + IIS). Slow column chromatographic separations of extract IIW + IIS (SiO_2, $CHCl_3$: CH_3OH) were found to be unsuccessful due to intractable mixtures obtained after elution.

Esterification of extract IIW + IIS(Ig) with ethereal diazomethane concentration and purification by slow column

chromatography (SiO_2, gradient (1-10% CH_3OH in $CHCl_3$) afforded component J (10 mg) as one of the medium polarity fractions. Component J is a white solid, melting point > 300°C.

TLC: Rf 0.43 ($CHCl_3$: CH_3OH, 93:7).

HREIMS (Probe, 250°C) m/z (mol.for., rel.int.): $C_{35}H_{50}O_7$ [M^+, Calcd:582.3543; found 582.3544](9), $C_{34}H_{47}O_7$(567, 13, M^+-CH_3),$C_{35}H_{48}O_6$ (564, 4, M^+-H_2O), $C_{35}H_{45}O_6$ (549, 6, $M^+-CH_3-H_2O$), $C_{32}H_{47}O_5$ (511, 8), $C_{30}H_{43}O_4$ (467, 16),$C_{31}H_{44}O_3$ (464, 9, M^+-2 $COOCH_3$), $C_{30}H_{43}O_3$ (451, 22), $C_{30}H_{42}O_3$ (450, 33), $C_{30}H_{41}O_3$ (449, 100), $C_{30}H_{43}O_2$ (435, 22), $C_{30}H_{39}O_2$ (431, 28), $C_{23}H_{31}$ (307, 16) $C_{21}H_{27}$(279, 8), $C_{13}H_{17}$ (221, 10), $C_{16}H_{21}$ (213, 11) $C_{14}H_{19}$ (187, 14), $C_{14}H_{17}$(185, 12), $C_{13}H_{15}$ (171,11) $C_{12}H_{15}$ (159, 14), $C_{12}H_{13}$ (157, 11), $C_9H_{13}O_2$(153, 11) $C_9H_{12}O_2$ (152, 11), $C_9H_{11}O_2$(151, 57), $C_{11}H_{13}$ (145, 14) $C_{10}H_{15}$(135, 12), $C_{10}H_{11}$(107, 15) C_8H_9 (105, 14), C_7H_{11}(95, 15) C_7H_9(93, 11), C_7H_7(91, 11), C_6H_9 (81, 12), C_5H_9(69, 13), and C_4H_7(55, 19).

CIMS m/z (rel, int): 600 (15.4)

FTIR ($CHCl_3$ cast): 3600-2400 (b) peak at 3480 cm^{-1}, 2951(b), 2876 (s), 2830 (m) 1732 (b, s, COOR) (1710, 1680, 1650 sh.), 1454 (w), 1435 (w), 1380 (m), 1267 (m), 1234 (m), 1202 (m), 1157 (w), 1143 (w), and 754 (m)cm^{-1}.

^1H NMR (400.1 MHz, $CDCl_3$) δ: 6.24 (br,s, 1H), 5.97 (br.s, 1H), 4.80 (br.s, 2H), 4.10 (m, 2H), 3.72 (br.s, 3H), 3.42 (br.s, 3H), 2.88 (dd, J=4 and 15 Hz, 1H), 2.56 (m, 2H), 1.36 (d, J=7.5 Hz, 3H), 1.00 (br.s, 6H), 0.94 (S, 3H), 0.92 (S,

3H), 0.88 (br.s, 3H) and 0.72 (br.S, 3H).

4.4.2 Acetylation of component J

Compound J (5 mg) was acetylated with acetic anhydride (0.5 mL) and pyridine (0.5 mL) for 24 hours. A complex mixture of inseparable products, which could not be analysed, was obtained.

4.5 Separation of the metabolites from extract IIb

4.5.1 Isolation of component K

Extract IIb (1.5 g), comprising a complex mixture of as many as 14 components (TLC), was subjected to slow chromatography over silica gel (column diameter = 35 mm, SiO_2 depth = 40 cm, using Skellysolve B (Table 8) or chloroform (Table 9) as packing and first eluting solvent.

Normal gravity conditions with an elution rate of 30 mL/hr were used. Component K (8 mg and 30 mg) was isolated according to the elution scheme described in Table 8 and Table 9 respectively. The isolation of component K by a chromatographic procedure was very difficult since closely related inseparable compounds co-eluted with component K. It was found that careful attention to several experimental conditions during the chromatographic procedure allowed successful purification of this compound. These include the following:

1. the flow rate for elution 30 mL/h gave good results.

2. column preparation (packing with the eluting solvent ($CHCl_3$: CH_3OH, 99:1) gave mixtures.

3. immediate monitoring of the eluants by TLC (allowed the adjustment of the eluant to the appropriate mixture of solvents).

4.5.2 Identification of component K as polyporenic acid-C (47)

TLC: Rf. 0.17 ($CHCl_3$: CH_3OH, 99:1)
0.23 ($CHCl_3$: CH_3OH, 96.5:3.5)
0.38 ($CHCl_3$: CH_3OH, 92.5:7.5)
0.45 ($CHCl_3$: CH_3OH, 90:10) and
0.15 Skellysolve B - acetone (4:1).

MP: 268 - 272°C, change in crystal shape at 245-250°C on melting.

$[\alpha]_D^{25}$ = +13.75 (c = 0.0008 g/ml, $CHCl_3$)

Mol. rot. [ϕ]: α x m/100 = 66.27

ORD: Plain positive curve. It does not rise appreciably with decreasing λ.

CD: $[\theta]_{308nm}$, -483.78 (c = 1.5 mg/ml, l= 1mm, scale = 1 millidegree/cm, solvent= $CHCl_3$).

UV λmax. (CH_3OH) : 237 (logϵ 4.15), 244 (logϵ 4.22) and 252 (logϵ 4.05) nm.

HREIMS (Probe, 250°C) m/z (mol. for., rel. int): $C_{31}H_{46}O_6$ [M$^+$,Calcd: 482.3384; found 482.3385] (16), 467 ($C_{30}H_{43}O_6$,10), 464 ($C_{31}H_{44}O_3$, 12), 449 ($C_{30}H_{41}O_3$, 38), 437

($C_{30}H_{45}O_2$, 3), 368 ($C_{24}H_{32}O_3$, 4), 327, ($C_{22}H_{31}O_2$, 3), 311 ($C_{21}H_{27}O_2$, 4), 310 ($C_{22}H_{30}O$, 15), 309 ($C_{22}H_{29}O$, 54), 308 ($C_{22}H_{28}O$, 19), 295 ($C_{21}H_{27}O$, 19), 294 ($C_{21}H_{26}O$, 26), 293 ($C_{21}H_{25}O$, 100) 271 ($C_{19}H_{27}O$, 10), 270 ($C_{19}H_{26}O$, 21), 269 ($C_{19}H_{25}O$, 37), 268 ($C_{19}H_{24}O$, 18), 253 ($C_{19}H_{25}$, 10), 96 (C_7H_{12}, 7), 84 (C_6H_{12}, 3), 69 (C_5H_9, 22) and 55 (C_4H_7, 43).

FTIR ($CHCl_3$ cast): 3600-2400 (b), peak at 3350 (m), 3040(w), 2960 (s), 2933 (m), 2872 (m), 2853 (m), 1714 (s), 1680 (s), 1640 (w), 1450 (m), 1395 (m), 1380 (w), 1310 (w), 1250 (s), 1200 (m), 1170 (w), 1138 (w), 1120 (s), 1090 (m), 1065 (m), 1025 (m), 1000 (w), 935 (w), 925 (w), 890 (m), 840 (w), 790 (w), 654 (w) and 615 (w) cm^{-1}.

^1H NMR (400.1 MHz, $CDCl_3$) δ: 5.52 (br.d, J=7 Hz, 1H), 5.37 (br.d, J=7 Hz, 1H), 4.8 (br.s, 1H), 4.74 (br.s 1H), 4.15 (ddd, J=1.5, 5.5, and 9 Hz, 1H), 2.78 (ddd, J=6, 14 and 14 Hz, 1H), 2.52 (dt, J=3.0 and 11 Hz, 1H) 2.36 (m, 1H), 2.3-1.6 (cm's,16H), 1.55 (dd, J=3.5 and 11Hz, 1H), 1.19 (s, 3H), 1.14 (s, 3H), 1.12 (s, 3H), 1.03 (d, J=7 Hz, 3H), 1.01 (d, J=7 Hz, 3H), and 0.66 (s, 3H, C-18 $C\underline{H}_3$)

^{13}C NMR (100.61 MHz, $CDCl_3$:CD_3OD) δ: 217.6 (s), 178.7 (s), 155.4 (s), 144.3 (s), 142.2 (s), 120.5 (d), 117.2 (d), 106.7 (t), 76.7 (d), 56.9 (d), 50.7 (d), 48.8 (s), 47.6 (s), 46.9 (d), 44.8 (s), 43.3 (t), 37.4 (s), 36.7 (t), 35.5 (t), 34.9 (t), 34.0 (d), 32.3 (t), 30.5 (t), 26.0 (q), 25.4 (q), 23.7 (t), 22.5 (q), 22.1 (q), 21.9 (q), 21.8 (q), and 17.2 (q).

4.5.3 Methyl polyporenate-C (47a)

Polyporenic acid-C (2 mg, 4.1×10^{-3} mmoles) was dissolved in diethyl ether (1 mL) and diazomethane in diethyl ether (2 mL) were added dropwise to the stirred solution at room temperature. A brisk reaction occured with the evolution of a gas. The TLC of the reaction mixture showed complete conversion to the product within half an hour. The reaction mixture was concentrated to give a quantitative yield of methyl polyporenate-C (47a).

TLC: Rf 0.52 ($CHCl_3$: CH_3OH, 96.5:3.5)

MP: 166-172°C, can be raised to 190°C by repeated recrystallization from ($CHCl_3$: CH_3OH, 99:1).

$[\lambda]_D^{25}$: +10.0 (c = 0.0019 g/mL, $CHCl_3$)

Mol.rot $[\phi]$ = 49.6°

UV λmax.(CH_3OH): 236 (logϵ 4.08), 243(logϵ 4.15), and 252 (logϵ 3.99) nm.

HREIMS (Probe, 250°C) m/z (mol.for., rel. int.): $C_{32}H_{48}O_4$ [M^+, Calcd : 496.354; found 496.3550] (8) 481 ($C_{31}H_{45}O_4$, 19), 478 ($C_{32}H_{46}O_3$, 4), 465 ($C_{31}H_{45}O_3$, 18) 463 ($C_{31}H_{43}O_3$, 20), 418 ($C_{30}H_{42}O$, 2), 400 ($C_{25}H_{36}O_4$, 2) 382 ($C_{25}H_{34}O_3$, 3), 327 ($C_{22}H_{31}O_2$, 2), 310 ($C_{22}H_{30}O$, 14), 309 ($C_{22}H_{29}O$, 53), 308 ($C_{22}H_{28}O$, 26), 295 ($C_{21}H_{27}O$, 26), 294 ($C_{21}H_{26}O$, 27), 293 ($C_{21}H_{25}O$, 100), 271 ($C_{19}H_{27}O$, 9), 270 ($C_{19}H_{26}O$, 23), 269 ($C_{19}H_5O$, 26), 268 ($C_{19}H_{24}O$, 19) 227 ($C_{13}H_{23}O_3$, 8), and 96 (C_7H_{12}, 3).

CIMS m/z (rel.int.): 514 (15.2)

FTIR ($CHCl_3$ cast): 3420 (m), 2957 (s), 2851 (s), 2922 (s) ,

1727 (s), 1682 (s), 1639 (w), 1463 (s), 1377 (m), 1190 (w), 1160 (m), 1020 (w), 890 (m), and 718 (m) cm^{-1}

^1H NMR (400.1 MHz, $CDCl_3$) δ: 5.51 (br.d, J=6 Hz, 1H), 5.39 (br.d, J=6 Hz, 1H), 4.78 (br.s, 1H), 4.72 (br.s, 1H), 4.13 (m, J=7 Hz, 1H), 3.70 (s, 3H), 2.78 (ddd, J=6, 15 and 15 Hz, 1H), 2.52 (dt, J=2.5 and 11 Hz, 1H), 2.36 (dt, J=4 and 14 Hz, 1H),2.14-2.3 (cm's), 2.08 (ddd, J=3.5, 7 and 15 Hz, 1H), 2.02-1.70 (cm's), 1.62 (dd, J=6 and 17 Hz, 1H) 1.54 (dd, J=3 and 11 Hz, 1H), 1.18 (s, 3H), 1.14 (s, 3H), 1.12 (s, 3H), 1.09 (s, 3H), 1.03 (d, J=7 Hz, 3H), 1.01 (d, J=7 Hz, 3H), and 0.62 (s, 3H, C-18 $C\underline{H}_3$)

^{13}C NMR (100.61 MHz, $CDCl_3$): 216.4 (s), 177.4 (s), 155.1 (s), 144.3 (s), 141.9 (s), 120.6 (d), 116.9 (d), 107.0 (t), 76.7 (d), 57.3 (d), 51.2 (q), 50.7 (d), 48.7 (s), 47.4 (s), 46.8 (d), 44.7 (s), 43.6 (t), 37.3 (s), 36.6 (t), 35.4 (t), 34.8 (t), 33.8 (d), 32.3 (t), 30.6 (t), 26.0 (q), 25.5 (q), 23.7 (t), 22.5 (q), 22.0 (q), 21.9 (q), 21.8 (q), and 17.2 (q).

4.5.4 O-Acetylpolyporenic acid-C (47b)

Polyporenic acid-C (2.5 mg, 5.1 x 10^{-3} mmoles) was dissolved in dry pyridine (1.5 mL) and acetic anhydride (2.0 mL) was added. The reaction was stirred at room temperature for 3 hours. The TLC of the reaction mixture showed complete conversion to the product within 2 hours. The reaction mixture was quenched with ice cold water (10 mL) and extracted with ethyl acetate. The ethyl acetate extract was

treated successively with 5% HCl (2x10 mL), 5% NaOH (2x10 mL), and brine (2x5 mL), then dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give O-acetylpolyporenic acid-C (47b) in quantitative yield.

TLC: Rf 0.22 ($CHCl_3$: CH_3OH, 99:1)

$[\lambda]_D^{25}$: -10° (c=1.8 mg/ml, $CHCl_3$)

Mol.rot: $[\phi]$ = -52.4°

UV λmax.(CH_3OH): 236 (logϵ3.96), 244 (logϵ 4.02), and 251 (logϵ 3.92) nm.

HREIMS (Probe, 250°C) m/z (mol. for., rel. int.) : $C_{33}H_{48}O_5$ [M$^+$, Calcd : 524.3489; found: 524.3470] (9), 464 ($C_{31}H_{44}O_3$, 31), 449 ($C_{30}H_{41}O_3$, 21), 368 ($C_{28}H_{32}O_3$, 4) 310 ($C_{22}H_{30}O$, 18), 309 ($C_{22}H_{29}O$, 54), 308 ($C_{22}H_{28}O$, 20), 295 ($C_{21}H_{27}O$, 28), 294 ($C_{21}H_{26}O$, 26), 293 ($C_{21}H_{25}O$, 100), 269 ($C_{19}H_{25}O$, 20), 268 ($C_{19}H_{24}O$ 18), 253 ($C_{19}H_{25}$, 5), 96 (C_7H_{12}, 5), 69 (C_5H_9,21), and 55 (C_4H_7,27).

CIMS (m/z, rel. int.) 542 (100).

FTIR ($CHCl_3$ cast): 3200-2400(b), 3030 (w), 2958 (s), 921 (s), 2850 (s), 1732 (s), 1700 (s), 1640 (w), 1463 (m), 1378 (m), 1366 (m), 1245 (m), 1113 (w), 1075 (w), 1022 (m), 968 (w), 940 (w), 925 (w), 895 (w), 815 (w), 796 (w), 720 (w), and 615 (w) cm^{-1}.

^1H NMR (400.1 MHz, $CDCl_3$) δ: 5.49 (br.d J=7 Hz, 1H), 5.37 (br.d, J=7 Hz, 1H), 4.99 (ddd, J=1.5, 5.5 and 8 Hz, 1H), 4.76 (d, J=1 Hz, 1H), 4.63 (d, J=1 Hz, 1H), 2.77 (ddd, J=5, 14, and 14 Hz, 1H), 2.7-2.1 (cm's), 2.06 (s, 3H, $OCOCH_3$), 2.0-1.3 (cm's), 1.19 (s, 3H), 1.13 (s, 3H), 1.10 (s, 3H),

1.05 (s, 3H), 1.02 (d, J=7 Hz, 3H), 1.01 (d), J=7 Hz, 3H) and 0.69 (s, 3H).

4.5.5 Methyl O-acetylpolyporenate-C (47c)

Methyl polyporenate-C (2.5 mg, 5.1 x 10^{-3} mmoles) was dissolved in dry pyridine (1 mL) and acetic anhydride (2.0 mL) was added. The TLC of the reaction mixture showed complete conversion to the product within 1 hour. Work up proceeded in the manner described for the preparation of O-acetylpolyporenic acid-C. Treatment of 47b (2mg) with ethereal diazomethane (2mL) followed by concentration gave methyl O-acetylpolyporenate-C (47c) in quantitative yield.

TLC: Rf 0.19 ($CHCl_3$) and 0.38 ($CHCl_3$: CH_3OH, 96.5:3.5)

UV λmax.(CH_3OH): 235 (logϵ 4.08), 244 (logϵ 4.16), and 251 (logϵ 4.00) nm.

HREIMS (Probe, 250°C) m/z, (mol.for., rel. int.): $C_{34}H_{50}O_5$, [M^+, Calcd : 538.3645; found: 538.3654] (15), 478 ($C_{32}H_{46}O_3$, 28), 465 ($C_{31}H_{45}O_3$, 28), 463 ($C_{31}H_{43}O_3$, 37) 382 ($C_{25}H_{34}O_3$, 3), 367 ($C_{24}H_{31}O_3$, 2), 310 ($C_{22}H_{30}O$, 21), 309 ($C_{22}H_{29}O$, 64), 308 ($C_{22}H_{28}O$, 27), 295 ($C_{21}H_{27}O$, 25), 294 ($C_{21}H_{28}O$, 27), 293 ($C_{21}H_{25}O$, 100) 268 ($C_{19}H_{24}O$, 25), 253 ($C_{18}H_{25}$, 2), and 96 (C_7H_{12}, 3).

FTIR ($CHCl_3$ cast): 3040 (w), 2960 (w), 2945 (s), 2850 (m), 1735 (s), 1709 (m), 1638 (w), 1580 (w), 1440 (w,d), 1380 (m), 1365 (w), 1244 (s), 1195 (w), 1160 (m), 1118 (w), 1060 (m), 1022 (m), 895 (w), 840 (w), 810 (w), 720 (w), 640 (w) and 600 (w) cm^{-1}.

^{1}H NMR (400.1 MHz, $CDCl_3$) δ: 5.49 (br.d, J=6 Hz, 1H), 5.39 (br.d, J=6 Hz, 1H), 4.98 (ddd, J=1.5, 6 and 8 Hz, 1H), 4.76 (br.s, 1H), 4.62 (d, J=1 Hz, 1H), 3.73 (s, 3H - $COOCH_3$), 2.78 (ddd, J=5, 14 and 14 Hz, 1H), 2.52 (cm. 1H), 2.06 (s, 3H, $OCOCH_3$), 1.19 (s, 3H), 1.12 (s, 3H), 1.10 (s, 3H), 1.05 (s, 3H), 1.02 (d, J=7 Hz, 3H), 1.00 (d, J=7 Hz, 3H), and 0.64 (s, 3H, C-18 CH_3).

4.5.6 Preparation of tetrakis(pyridine)silver dichromate ($Py_4Ag_2Cr_2O_7$)

$K_2Cr_2O_7$ (1g) was dissolved in H_2O (4 mL) and $AgNO_3$ (1.2 g) was added. Over the next 10 minutes, the solution was stirred and H_2O (4 mL) and pyridine (6 mL) were added. An orange yellow precipate was formed and collected by vacuum filtration. The solid was washed with benzene (3x10 mL) and the reagent dried overnight under vacuum to give $Py_4Ag_2Cr_2O_7$ in quantitative yield mp 110-156°C (decomp.). It is a stable reagent and has limited solubility in benzene.

4.5.7 Oxidation of polyporenic acid-C (47)

Polyporenic acid-C (2 mg, 4.14×10^{-3} mmoles) was dissolved in dry benzene (2 mL). An excess of $Py_4Ag_2Cr_2O_7$ (5 mg) was added to the reaction flask and the stirred mixture was heated at reflux under an atmosphere of N_2 for 4 hrs. TLC analysis of the reaction mixture showed an oxidation product with an Rf value higher than the original acid. The reaction mixture was filtered and the solid residue was

washed with cold benzene (50 mL). The solvent was evaporated and the product was purified by PTLC using $CHCl_3$: CH_3OH, 95:5 as eluant to remove the pyridinium salt. The pyridinum salt was retained at the base line of PTLC and a very low (25%) yield of the 16-oxopolyporenic acid-C (47d) was obtained.

TLC: Rf 0.30 ($CHCl_3$: CH_3OH, 96.5:3.5)

UV λmax.(CH_3OH): 234 (ε 11500), 242 (ε 12520), 251 (ε 10525), and 384 (ε 78) nm.

HREIMS (Probe, 200°C) m/z, (mol.for.,rel.int.) : $C_{31}H_{44}O_4$ [M$^+$, calcd; 480.3228; found 480.3233] (24), 465 ($C_{30}H_{41}O_4$, 16), 383 ($C_{24}H_{31}O_4$, 7), 313 ($C_{21}H_{29}O_2$, 20), 312 ($C_{21}H_{28}O_2$, 22), 311 ($C_{21}H_{27}O_2$, 100), 295 ($C_{21}H_{27}O$, 15), 270 ($C_{19}H_{26}O$, 20), 269 ($C_{19}H_{25}O$, 37), 96 (C_7H_{12}, 15), 69 (C_5H_9, 34), 67 (C_5H_7, 20), 57 (C_4H_9, 26), and 55 (C_4H_7, 61).

FTIR ($CHCl_3$ cast) : 3600-3200 (b), 2962 (s), 2933 (s), 2872 (s), 2853 (m), 1737 (m), 1707 (s), 1640 (w), 1595 (w), 1450 (m), 1398 (w), 1380 (w), 1290 (w), 1258 (m), 1230 (w), 1178 (m), 1140 (m), 1110 (m), 1075 (m), 1032 (w), 1036 (w), and 885 (m) cm^{-1}.

4.5.8 Oxidation of methyl polyporenate-C (47a)

Methyl polyporenate-C (3 mg, 6.2×10^{-3}mmoles) was dissolved in dry benzene (3.5 mL) and $Py_4Ag_2Cr_2O_7$ (4 mg) was added. The resulting solution was stirred and heated under reflux for 4 hrs. The hot solution was filtered through Celite-silica gel (1:1, 3 g), in a sintered funnel (4-8 μ,

porosity). The solid mass was washed with hot benzene (4x10 mL) to recover most of the oxidized product. The benzene extracts were combined and concentrated under reduced pressure to give methyl 16-oxo-polyporenate-C (47e) in 83% yield.

TLC: Rf 0.77 ($CHCl_3$: CH_3OH, 96.5:3.5)

HREIMS (Probe, 220°C) m/z (mol.for., rel. int.): $C_{32}H_{46}O_4$ [M^+, Calcd : 494.3384; found: 494.3381] (10), 479 ($C_{31}H_{43}O_4$, 29), 447 ($C_{30}H_{39}O_3$, 29), 398 ($C_{25}H_{34}O_4$, 7), 325 ($C_{22}H_{29}O_2$), 5), 313 ($C_{21}H_{29}O_2$, 24), 311 ($C_{21}H_{27}O_2$, 100), 270 ($C_{19}H_{26}O$, 18), 269 ($C_{19}H_{25}O$, 41), 171 ($C_{13}H_{15}$, 20), 157 ($C_{12}H_{13}$, 19), 96 (C_7H_{12}, 18), 81 (C_6H_9, 21), 69 (C_5H_9, 35), 67 (C_5H_7, 20) and 55 (C_4H_7, 58).

FTIR (CH_2Cl_2 cast): 3030 (w), 2963 (s), 2888 (s), 1735 (b,s), 1709 (s), 1640 (vw), 1453 (b,m), 1432 (m), 1383 (m), 1362 (w), 1347 (vw), 1310 (w), 1274 (w), 1254 (m,b), 1225 (w), 1210 (w), 1198 (w), 1156 (m,b), 1112 (m), 1050 (w), 1028 (w), 1002 (w), 998 (w), 970 (w), 950 (w), 892 (b,m), 808 (w), 735 (w), 660 (w), cm^{-1}.

^1H NMR (400.1 MHz, $CDCl_3$): δ5.56 (br.d, J=6.5 Hz, 1H), 5.50 (br.d, J=6.5 Hz, 1H), 4.78 (s, 1H), 4.74 (d, J=1.5 Hz, 1H), 3.72 (s,-$COOCH_3$ 2.78 (ddd, J=5.5, 11.5 and 12.5 Hz, 1H), 2.57 (dt, J=3.5 and 11Hz, 1H), 1.96 (dt, J=5.5 and 10Hz, 2H), 2.5-1.4 (cm's), 1.22 (s,3H) 1.15 (s, 3H), 1.11 (s, 3H), 1.07 (s, 3H), 1.01 (d, J=7 Hz, 3H), 1.00 (d, J=7 Hz, 3 , and 0.71 (s, 3H).

4.5.9 Lactonization of 47

Polyporenic acid-C (5 mg, 1.03×10^{-2} mmoles) was dissolved in benzene (2 mL) and excess p-toluenesulphonic acid (10 mg) in benzene (1 mL) was added to the stirred solution at room temperature. The solution was heated under reflux on a water bath. The TLC of the reaction mixture showed 3 overlapping spots. The reaction mixture was quenched with saturated solution of $NaHCO_3$ (2x4 mL). The benzene extract was concentrated and a mixture of lactones 47J (4 mg) was obtained.

FTIR ($CHCl_3$ cast). 2918 (s), 2981 (s), 2849 (s), 1767 (s), 1747 (s), 1707 (s), 1638 (m), 1550 (w), 1450 (m), 1376 (m), 1350 (w), 1300 (w), 1260 (m), 1194 (m), 1168 (m), 1092 (m), 1038 (m,b), 800 (m), 735 (w) and 700 (w).

4.5.10 Preparation of the dehydrating reagent (carboxy sulfamoyltriethylammonium hydroxide inner salt methyl ester)

Anhydrous methanol (1.12 g, 0.035 mol.) in benzene (dried over KOH, 2 mL) was added dropwise to a solution of chlorosulfonyl isocyanate (3.44 g, 0.0246 mol.) in dry benzene (10 mL) contained in a 50 mL round bottomed flask fitted with a 25 mL addition funnel. The exothermic reaction was controlled with a cool water bath. After the addition was complete (30 mintues), the solvent and excess methanol was removed from the reaction mixture under reduced pressure. The resulting white, crystalline mass was

recrystallized from toluene to give colorless needles of carbomethoxysulfamoyl chloride ($C_2H_4ClNO_4S$, 3.1g, mp 69°C lit.70-71°C).

Carbomethoxysulfamoyl chloride (3.1g, 0.0178 mol.) dissolved in benzene (10 mL) was added dropwise to a solution of Et_3N (4 g, 0.0396 mol.) in benzene (5 mL) under a N_2 atmosphere at ambient temperature. After the addition was complete (30 min.), the precipitate of triethylamine hydrochloride was removed by filtration and the solvent was evaporated under reduced pressure to afford a residual colorless oil which solidified on standing. Crystallization from toluene yielded colorless needles of carboxysulfamoyltriethylammonium hydroxide inner salt methyl ester ($CH_3O_2CNSO_2NEt_3$, 3.2 g, mp 70°C lit.71-72°C).
^1H NMR (80 MHz, $CDCl_3$) δ: 3.66 (s, 3H), 3.29 (q, J=7 Hz, 6H), and 1.15 (t, J=7 Hz, 9H).

4.5.11 Dehydration of polyporenic acid-C (47)

Polyporenic acid-C (1mg, 2.07×10^{-3} mmoles) was dissolved in dry benzene (2mL) and alkyl-N-carbomethoxysulfamate ester ($CH_3O_2CNSO_2NEt_3$, 1mg, 4.2mmoles) was added. The reaction was heated under reflux for 8 hours in an atmosphere of dry N_2. The reaction mixture was quenched with water (10 mL) and the benzene layer separated, dried (anhydrous Na_2SO_4) and concentrated under reduced pressure to yield a white solid (1 mg). The TLC of the reaction product showed two equal intensity nearly

overlapping spots corresponding to the possible elimination products of the dehydration reaction. Attempts to separate the two compounds failed. This was due to the similarity in Rf of the components and the instability of the resulting dehydration products, namely,

3-oxoeburico-7,9'',15'',24''-tetraene (47h) and

3-oxoeburico-7,9'',16'',24''-tetraene (47ha).

TLC: Rf. 0.37 and 0.38 (Skellysolve B: acetone, 4:1)

HREIMS (Probe, 200°C) m/z(mol. for., rel. int.): $C_{31}H_{48}O_3$ [M^+, Calcd : 464.3279; found: 464.3290] (94), 449 ($C_{30}H_{41}O_3$, 33), 381 ($C_{25}H_{33}O_3$, 58), 380 ($C_{25}H_{32}O_3$, 34) 368 ($C_{24}H_{32}O_3$, 17), 310 ($C_{22}H_{30}O$, 15), 309 ($C_{22}H_{29}O$, 39), 295 ($C_{21}H_{27}O$, 36), 294 ($C_{21}H_{26}O$, 16), 293 ($C_{21}H_{25}O$, 62), 270 ($C_{19}H_{26}O$, 22), 269 ($C_{19}H_{25}O$, 60), 268 ($C_{19}H_{24}O$, 12), 183 ($C_{14}H_{15}$, 21), 171 ($C_{13}H_{15}$, 33), 157 ($C_{12}H_{13}$, 35), 145 ($C_{11}H_{13}$, 30), 143 ($C_{11}H_{11}$, 24), 133 ($C_{10}H_{13}$, 24), 131 ($C_{10}H_{11}$, 22), 121 (C_9H_{13}, 20), 119 (C_9H_{11}, 36), 109 (C_8H_{13}, 38), 107 (C_8H_{11}, 31), 105 (C_8H_9, 37), 97 (C_7H_{13}, 77), 96 (C_7H_{12}, 53), 95 (C_7H_{12}, 53), 95 (C_7H_{13}, 85), 84 (C_6H_{12}, 49), and 83 (C_6H_{11}, 100).

FTIR ($CHCl_3$ cast): 3600-2200 (b) peak at 3380 (m), 2955 (s), 2922 (s), 2870 (m), 2850 (m), 1710 (m, b), 1640 (vw), 1460 (m,b) 1380 (m,b), 1260 (m), 1200-920 (b), 895 (w,b) and 620 (m) cm^{-1}.

4.5.12 Lithium aluminium hydride reduction of methyl polyporenate-C (47a)

Methyl polyporenate-C (3mg, 6.0×10^{-3}mmoles) was added carefully to a cooled (0°) suspension of excess lithium aluminium hydride (5mg) in tetrahydrofuran, dried over K, 3mL). Dry THF (1mL) was added and the mixture was stirred for 20 minutes under an atmosphere of argon. The cooling bath was removed and the reaction stirred for further 2.5 hrs. During this period, the progress of the reaction was monitored by TLC. The reaction mixture was then recooled to 0°C and excess of the reagent was destroyed by excess of ethyl acetate (3mL). After stirring for 10 minutes, saturated aqueous NH_4Cl(3.5 mL) was added, the cooling bath was removed and the mixture was stirred for 25 minutes. The reaction mixture was filtered through Celite (2g) contained in a sintered funnel (4-8μ porosity). The Celite was washed with ethyl acetate (3x5 mL). The organic filtrates were combined, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to give the reduction product i.e., 3β,16α-21-trihydroxyeburico-7,9'',24''-triene (47f) (2.7 mg) as white solid in high purity which was used without further purification.

TLC: Rf. 0.19 ($CHCl_3$: CH_3OH, 96.5:3.5)

HREIMS (Probe, 220°C) m/z (mol. for., rel. int.): $C_{31}H_{50}O_3$ [M$^+$, Calcd; 470.3747; found: 470.3759] (29), 455 ($C_{30}H_{47}O_3$, 28), 439 ($C_{30}H_{47}O_2$, 37), 437 ($C_{30}H_{45}O_2$, 22), 419 ($C_{30}H_{43}O$, 9), 356 ($C_{24}H_{36}O_2$, 8), 329 ($C_{22}H_{33}O_2$, 8), 312 ($C_{22}H_{32}O$, 40),

311 ($C_{22}H_{31}O$, 100), 310 ($C_{22}H_{30}O$, 20) 297 ($C_{21}H_{29}O$, 28), 295 ($C_{21}H_{27}O$, 38), 273 ($C_{19}H_{29}O$, 18), 272 ($C_{19}H_{28}O$, 18), 271 ($C_{19}H_{27}O$, 47), 246 ($C_{17}H_{26}O$, 20), 239 ($C_{18}H_{23}$, 21), 237 ($C_{18}H_{21}$, 20), 227 ($C_{17}H_{23}$, 20), 225 ($C_{17}H_{21}$, 25), However, hydrocarbon unit fragment peaks at the following amu's although insignificant occur at intensites greater than 20% relative to base peak. They include fragments at 211, 199, 197, 187, 185, 183, 173, 172, 171, 170, 169, 160, 159, 158, 157, 147, 145, 143, 135, 133, 132, 131, 123, 121, 119, 109, 107, 105, 97, 95, 93, 91, 83, 81, 79, 71, 69, 67, 57, and 55.

FTIR ($CHCl_3$ cast): 3700-2100 (b) peak at 3295 (s), 2961 (s), 2980 (s), 1640 (m), 1450 (m), 1372 (m), 1188 (w), 1149 (w), 1070 (m), 1036 (m), 990 (m), 889 (m), and 850 (w) cm^{-1}.

^1H NMR (400.1 MHz, $CDCl_3$) δ: 5.49 (d, J=7 Hz, 1H), 5.36 (d, J=7 Hz, 1H) 4.76 (br.s, 1H), 4.72 (d, J=1.8 Hz, 1H), 4.25 (ddd, J=1.5, 6, 8.5 Hz, 1H), 3.86 (dd. J=2 and 11 Hz, 1H), 3.58 (dd, J=2.5 and 11 Hz, 1H), 3.25 (dd, J=4.5 and 11 Hz, 1H), 2.45 (br. signal, disappears on D_2O addition), 1.45 (dt, J=3.5 and 12 Hz, 2H), 1.10 (s, 3H), 1.03 (s, 3H), 1.01 (d, J=6.5 Hz, 3H), 1.00 (d, J=6.5 Hz, 3H), 0.97 (s, 3H), 0.88(s, 3H), and 0.55 (s, 3H).

4.5.13 Correlation experiment number I

4.5.13.1 Sodium borohydride reduction of polyporenic acid-C (47)

Polyporenic acid-C (2.1mg, 4.3×10^{-3}mmoles) was dissolved in methanol (2mL) and excess of $NaBH_4$ (5mg) was added in two portions. The resulting solution was stirred at room temperature. After 1 hour, TLC analysis showed complete conversion to product. The reaction mixture was quenched by adding excess acetic acid (2mL). Further dichloromethane (10 mL) and water (10mL) were added. The organic and water layer were separated. The water layer was extracted with CH_2Cl_2 and the organic extracts were combined, dried over anhydrous Na_2SO_4, filtered and concentrated to dryness to give of the reduction product (1.9mg), i.e., 3β, 16α-dihydroxyeburico-7,9'', 24''-triene (48).

TLC: Rf 0.26 ($CHCl_3$: CH_3OH, 92.5:7.5) and 0.32 ($CHCl_3$: CH_3OH, 90:10)

UV λmax.(CH_3OH): 235 (ε10220), 243 (ε3450) and 251 (ε11200) nm.

HREIMS (Probe, 250°C) m/z, (mol. for., rel. int.): $C_{31}H_{48}O_4$ [M^+, Calcd: 484.3540; found: 484.3530] (13), 469 ($C_{30}H_{45}O_4$, 10), 466 ($C_{31}H_{46}O_3$, 14), 453 ($C_{30}H_{45}O_3$, 22), 451 ($C_{30}H_{43}O_3$, 20), 435 ($C_{30}H_{43}O_2$, 23), 433 ($C_{30}H_{41}O_2$, 16), 339 ($C_{23}H_{31}O_2$, 10), 311 ($C_{22}H_{31}O$, 26), 297 ($C_{21}H_{29}O$, 18), 296 ($C_{21}H_{28}O$, 20), 295 ($C_{21}H_{27}O$, 54), and 277 ($C_{21}H_{25}$, 14).

However, hydrocarbon unit fragment peaks at the following amu's occur at intensities greater than 20% relative to base

peak. They include fragments at 159, 145, 121, 119, 109, 107, 105, 97, 95, 93, 91, 83, 81, 71, 69, 67, 57 and 55.
FTIR ($CHCl_3$ cast): 3600-2400 (b) peak at 3450 (m), 3028 (w), 2960 (s), 2929 (s), 2870 (s), 1736 (s), 1640 (w), 1600 (v,w), 1446 (m), 1378 (m), 1288 (w), 1258 (w), 1195 (w), 1158 (m), 1090 (m), 1036 (m), and 889 (w) cm^{-1},
^1H NMR (400.1MHz, $CDCl_3$) δ: 5.48 (br.d, J=6 Hz,1H), 5.31 (br.d, J=6 Hz, 1H), 4.78 (br.s, 1H) 4.72 (br.s, 1H), 4.1 (cm, 1H), 3.2 (cm, 1H), 2.9-2.5 (cm's), 2.50 (ddd, J=3.0, 11.0, and 11 Hz, 1H), 2.4-1.2 (cm's), 1.11 (s, 3H), 1.04 (s, 3H), 1.01 (d, J=7 Hz, 3H), 1.00 (d, J=7 Hz, 3H), 0.97 (s, 3H), 0.89 (s, 3H), and 0.62 (s, 3H).

4.5.14 Correlation experiment number II

4.5.14.1 Sodium borohydride reduction of methyl polyporenate-C (47a)

The reduction of methyl polyporenate-C (2.5mg, 5.0×10^{-3} mmoles) was carried out by a similar procedure as described for the $NaBH_4$ reduction of polyporenic acid-C (47).
Methyl-3β, 16α-dihydroxyeburico 7,9'',24''trien-21-oate (48a), mp 160-170°, 2.3 (mg) was obtained as a white solid.
TLC: Rf 0.61 ($CHCl_3$: CH_3OH, 92.5:7.5)
HREIMS (Probe, 220°C) m/z (mol. for., rel. int.): $C_{32}H_{50}O_4$ [M^+ Calcd: 498.3696; found: 498.3715] (13), 483 ($C_{31}H_{47}O_4$, 12), 480 ($C_{32}H_{48}O_3$, 8), 467 ($C_{31}H_{47}O_3$, 16), 465 ($C_{31}H_{45}O_3$, 9), 447 ($C_{31}H_{43}O_2$, 8), 353 ($C_{24}H_{33}O_2$, 6), 311 ($C_{22}H_{31}O$, 47), 310 ($C_{22}H_{30}O$, 15), 296 ($C_{21}H_{28}O$, 24), 295 ($C_{21}H_{27}O$, 100),

277 ($C_{21}H_{25}$, 18), 272 $C_{18}H_{28}O$, 16), 96 (C_7H_{12}, 9), 69 (C_5H_9, 29), and 55 (C_4H_7, 43).

FTIR ($CHCl_3$ cast): 2960 (s), 2929 (s), 2870 (s), 1732 (s 1640 (w), 1600 (vw), 1450 (m, b), 1378 (m), 1285 (m), 1257 (m) 1195 (m), 1160 (m), 1095 (m), 1037 (m), 992 (w), 889 (w), and 755 (w).

^1H NMR (400.1 MHz, $CDCl_3$) δ: 5.48 (br.d, J=6.5 Hz, 1H), 5.31 (br.d, J=6.5 Hz, 1H), 4.78 (br.s, 1H), 4.72 (d, J=0.5 Hz, 1H), 13 (cm, 1H, Sharpens on deuteration to ddd (J=1.5, 5.5 and 9 Hz), 3.72 (S, 3H, $-COOCH_3$), 3.25 (m, 1H, sharpens on deuteration to dd (J=4.5 and 11.5 Hz, 1H), 2.50 (dt, J=3 and 11 Hz, 1H), 2.8-2.5 (cm's), 2.4-1.2 (cm's), 1.10(s, 3H), 1.03 (s, 3H), 1.01 (d, J=7 Hz, 3H), 1.00 (d, J=7 Hz, 3H), 0.97 (s, 3H), 0.89 (s, 3H), and 0.60 (s, 3H).

4.5.15 Isolation and identification of component L as dehydrotumulosic acid (48)

Further fractionation of extract IIb with higher polarity solvent ($CHCl_3$: CH_3OH, 96:4), gave impure component L (3.5 mg). Purification by centrifugal PTLC (Chromatotron; Skellysolve B: ethyl acetate : 2:1, 1:1, 1:2) afforded component L (1.5 mg) mp> 300°C. In an improved isolation scheme (see Table 9) component L was isolated as a mixture containing component K. Easy purification by PTLC using (5% CH_3OH in $CHCl_3$) gave component L(5 mg) as a white solid. Component L is one of the minor, more polar components of the above extract.

TLC: Rf 0.26 ($CHCl_3$: CH_3OH, 92.5:7.5) and 0.31 ($CHCl_3$: CH_3OH, 90:10).

UV λmax.(CH_3OH): 236 (logϵ 3.98), 243 (logϵ4.14), and 251 (logϵ 3.90) nm.

HREIMS (Probe, 250°C), m/z, (mol. for., rel. int.): $C_{31}H_{48}O_4$ [M$^+$, Calcd : 484.3540; found: 484.3548] (20), 469 ($C_{30}H_{45}O_4$, 9), 466 ($C_{31}H_{46}O_3$, 15), 453 ($C_{30}H_{45}O_3$, 24), 451 ($C_{30}H_{43}O_3$, 17), 433 ($C_{30}H_{41}O_2$, 15), 339 ($C_{23}H_{31}O_2$, 12), 311 ($C_{22}H_{31}O$, 26), 297 ($C_{21}H_{29}O$, 16), 296 ($C_{21}H_{28}O$, 20), 295 ($C_{21}H_{27}O$, 100), 277 ($C_{21}H_{25}$, 20), 272 ($C_{19}H_{28}O$, 19), 96 (C_7H_{12}, 6), 69 (C_5H_9, 27), and 55 (C_4H_7, 36).

FTIR ($CHCl_3$ cast): 3600-1900 (b) peak at 3380 (m). 2950 (s), 2920 (s), 2860 (s), 1735 (m), 1732 (m), 1640 (w), 1600 (bw), 1445 (m), 1375 (m), 1288 (w), 1248 (m), 1200 (w), 1165 (m), 1090 (w), 1025 (w) and 884 (w) cm^{-1}.

^{1}H NMR (400.1 MHz, $CDCl_3$) δ: 5.48 (m, 1H), 5.32 (m, 1H), 4.78 (br.s, 1H), 4.72 (br.s , 1H), 4.1 (m, 1H), 3.2 (m,1H), 2.52 (dt, J=3.0 and 11 Hz, 1H), 2.9-2.55 (cm's), 2.4-1.2 (cm's), 1.12 (s, 3H), 1.03 (s, 3H), 1.02 (d, J=6.5 Hz, 3H), 1.00 (d, J=6.5 Hz, 3H), 0.97 (s, 3H), 0.89 (s, 3H), and 0.61 (s, 3H).

4.5.16 Preparation of methyl dehydrotumulosate (48a)

Dehydrotumulosic acid (2 mg, 4.13×10^{-3} m.moles) was dissolved in diethyl ether (1 mL) and ethereal diazomethane (2 mL) was added dropwise to the stirred solution at room temperature. A brisk reaction occured with the evolution of

a gas. The TLC of the reaction mixture showed complete conversion to the product within 1 hr. The reaction mixture was concentrated to give a quantitative yield of methyl dehydrotumulosate (48a) as a white solid.

MP: 163-172°C, change in crystal shape to sharp needles occurs at 145°C.

TLC: Rf 0.61 ($CHCl_3$: CH_3OH, 92.5:7.5).

UV λmax.(CH_3OH): 235 (ε 12700), 242 (ε 14830), and 251 (ε 10200) nm.

$[\alpha]_D^{25}$: +7.5° (c=0.0008 g/ml, $CHCl_3$).

Mol. rot [φ]: +37.35

HREIMS (Probe 325°C) m/z (mol. for., rel. int.): $C_{32}H_{50}O_4$ [M⁺, Calcd : 498.3696; found 498.3706] (10), 483 ($C_{31}H_{47}O_4$, 7), 480 ($C_{32}H_{48}O_3$, 9), 467 ($C_{31}H_{47}O_3$, 12), 465 ($C_{31}H_{45}O_3$, 9), 447 ($C_{31}H_{43}O_2$, 10), 353 ($C_{24}H_{33}O_2$, 8), 311 ($C_{22}H_{31}O$, 40), 310 ($C_{22}H_{30}O$, 12), 296 ($C_{21}H_{28}O$, 28), 295 ($C_{21}H_{27}O$, 100), 277 ($C_{21}H_{25}$, 19), 96 (C_7H_{12} , 8), 81 (C_6H_9, 23), 69 (C_5H_9, 36), 57 (C_4H_9, 23), and 55 (C_4H_7, 54).

FTIR ($CHCl_3$ cast) : 3436 (b), 2959 (s), 2927 (s), 2862 (s), 1732 (s), 1640 (w), 1605 (vw), 1460 (b,m), 1378 (m), 1285 (m), 1257 (m), 1194 (m), 1159 (m), 1096 (m), 1037 (m), 992 (vw), 885 (w) and 752 (w) cm^{-1}.

^1H NMR (400.1 MHz, $CDCl_3$) δ: 5.48 (br.d, J=6.5 Hz, 1H), 5.30 (br.d, J=6.5 Hz, 1H), 4.78 (br.s, 1H), 4.72 (d, J=0.5 Hz, 1H), 4.13 (br.t, J=7.5 Hz, 1H), 3.71 (s, 3H, -COOC<u>H</u>$_3$), 3.26 (dd, J=4 and 11 Hz, 1H), 3.1-2.6 (cm's), 2.51 (dt, 3.0 and 11 Hz, 1H), 2.4-2.1 (cm's), 1.10 (s, 3H), 1.03 (s, 3H), 1.02

(d, J=7 Hz, 3H), 1.00 (d, J=7 Hz, 3H), 0.97 (s, 3H), 0.89 (s, 3H), and 0.60 (s, 3H).

^{13}CNMR (100.61 MHz, $CDCl_3$) δ: 176.1 (s), 155.1 (s), 145.7 (s), 141.6 (s), 121.0 (d), 115.8 (d), 107.0 (t), 78.9 (d), 77.0 (d), 57.4 (d), 51.2 (q), 49.2 (d), 48.6 (s), 46.7 (d), 44.7 (s), 43.6 (t), 38.7 (s), 37.5 (s), 35.7 (t), 35.4 (t), 33.8 (d), 32.3 (t), 30.6 (t), 28.2 (q), 27.8 (t), 26.2 (q), 23.0 (t), 22.7 (q), 21.9 (q), 21.8 (q), 17.2 (q), and 15.8 (q).

4.5.17 O-Diacetyldehydrotumulosic acid (48b)

Dehydrotumulosic acid (2.5 mg, 5.16×10^{-3} mmoles) was dissolved in dry pyridine (1.5mL) and acetic anhydride (1mL) was added. The reaction was stirred at room temperature for 8 hours. The TLC of the reaction mixture showed complete conversion to the product. After this time the pyridine was azeotropically removed with toluene. The residue was passed through a silica column (pasteur pipette, eluant $CHCl_3$: CH_3OH, 98:2, 2 cm column bed) and compound 48b (2mg) was obtained as a white to yellow semisolid which showed single spot on TLC in various solvent systems.

TLC: Rf 0.76 ($CHCl_3$: CH_3OH, 96.5:3.5).

HREIMS (Probe, 250°C) m/z (mol. for., rel.int.): $C_{35}H_{52}O_6$ [M^+, Calcd : 568.3750; found: 568.3765] (18), 508 ($C_{33}H_{48}O_4$, 35), 495 ($C_{32}H_{47}O_4$, 18), 433 ($C_{30}H_{41}O_2$, 35), 353 ($C_{24}H_{33}O_2$, 43), 339 ($C_{23}H_{31}O_2$, 24), 337 ($C_{23}H_{29}O_2$, 18). 295 ($C_{21}H_{27}O$, 39), 277 ($C_{21}H_{25}$, 36), 157 ($C_{12}H_{13}$, 22), 149 ($C_8H_5O_3$, 41),

96 (C_7H_{12}, 8), and 55 (C_4H_7, 100).

Other peaks which are 20% as intense as base peak are reported. Only amu of the fragments are mentioned. They are 119, 109, 107, 105, 97, 95, 93, 91,85,83, 81, 73, 71, 69, and 67.

FTIR ($CHCl_3$ cast): 3600-2400 (b), 2957 (s), 2924 (s), 2853 (s), 1733 (s), 1700 (m), 1640 (w), 1370 (m,d), 1244 (b,s), 1118 (w), 1030 (m,b), and 895 (w) cm^{-1}.

^1H NMR (400.1 MHz, $CDCl_3$) δ: 5.45 (br.d, J=6.5 Hz, 1H), 5.36 (br.d, J=6.5 Hz, 1H), 4.99 (m, 1H), 4.76 (br.s, 1H), 4.63 (br.s, 1H), 4.52 (cm, 1H), 2.9-2.08 (cm's) 2.06 (s, 3H, $-OCOC\underline{H}_3$), 2.05 (s, 3H, $-OCOC\underline{H}_3$), 2.02-1.10 (cm's), 1.04 (s, 3H), 1.03 (s, 3H), 1.02 (d, J=6 Hz, 3H), 1.01 (d, J=6 Hz, 3H), 0.96 (s,3H), 0.88 (s, 3H), and 0.63 (s, 3H).

4.5.18 Preparation of methyl O-diacetyldehydrotumulosate (48c)

Methyl dehydrotumulosate (1.5 mg, 3.01×10^{-3} mmoles) was treated with pyridine (1mL) and acetic anhydride (1.5mL) at room temperature for 4 hours. The reaction mixture was quenched with ice cold water (10mL) and extracted with ethyl acetate. The ethyl acetate extract was treated successively with 5% HCl (2x5 mL), 5% NaOH (2x5 mL) and brine (2x5 mL) then dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give (1mg) methyl O-diacetyl dehydrotumulosate (1 mg) (48c) as a single spot on TLC.

TLC: Rf 0.87 ($CHCl_3$: CH_3OH, 96.5:3.5)

HREIMS (Probe, 180°C) m/z (mol.for.,rel.int.): $C_{38}H_{54}O_6$ [M$^+$,Calcd: 582.3906 ; found: 582.3893] (9), 522 ($C_{34}H_{50}O_4$, 13), 509 ($C_{33}H_{49}O_4$, 16), 447 ($C_{31}H_{43}O_2$, 18), 396 ($C_{29}H_{48}$, 19), 353 ($C_{24}H_{33}O_2$, 46), 352 ($C_{24}H_{32}O_2$, 18), 339 ($C_{23}H_{31}O_2$, 11), 295 ($C_{21}H_{27}O$, 27), 277 ($C_{21}H_{25}$, 25), 149 ($C_8H_5H_3$, 48), 96 (C_7H_{12}, 8), 84 (C_6H_{12},16), 57 (C_4H_9, 100), 56 (C_4H_8, 22), and 55 (C_4H_7, 76).

FTIR ($CHCl_3$ cast): 2958 (sh.) 2917 (s), 2848 (s), 1737 (s), 1640 (w), 1461 (m,b), 1379 (m,b), 1242 (s), 1160 (m), 1030 (m), 895 (w) and 722 (w) cm^{-1}.

^1H NMR (400.1 MHz, $CDCl_3$) δ: 5.43 (br.d, J=6.5 Hz, 1H), 5.38 (br.d, J=6.5 Hz, 1H) 4.98 (m, 1H), 4.76 (br.s, 1H), 4.64 (br.s, 1H), 4.55 (m,1H), 3.71 (s, 3H,-COOC$\underline{H}_3$), 2.06 (s, 3H, -OCOC$\underline{H}_3$), 2.05 (s, 3H, -OCOC$\underline{H}_3$, 1.05 (s, 3H), 1.03 (s, 3H), 1.00 (d, J=6 Hz, 3H), 1.00 (d, J= 6 Hz, 3H), 0.97 (s, 3H), 0.88 (s, 3H), and 0.63 (s, 3H).

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